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Mobilization of Storage Protein Reserves in Radiata Pine (*Pinus radiata* D. Don) Seeds during Germination and Early Seedling Growth

by

Samuel Frederick Tom Fashu-Kanu

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science in Plant Biology

Department of Biological Sciences

Edmonton, Alberta
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University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Mobilization of Storage Protein Reserves in Radiata Pine (*Pinus radiata* D. Don) Seeds during Germination and Early Seedling Growth" submitted by Samuel Frederick Tom Fashu-Kanu in partial fulfillment of the requirements for the degree of Master of Science in Plant Biology.

TO MY SON

Abstract

This thesis deals with the effect of stratification on germination, identifies and characterizes storage protein reserves in radiata pine (*Pinus radiata* D. Don.) seeds and examines the changes that occur in these reserves during germination and early seedling growth. Over 50 % of radiata pine seed seeds were dormant and required a period of moist stratification at 2 °C in the dark to break dormancy and to ensure uniform growth. Seeds were stratified for 28 days and transferred to an incubator where they were imbibed at 27 °C with 19 μmol m^2 s^{-1} continuous lights. The mature radiata pine seed contained an average of 1.82 mg storage protein reserves. Approximately 93 % of the storage protein reserves were stored in the haploid megagametophyte. These storage protein reserves were classified according to their solubility in phosphate buffer as soluble and insoluble.

Interestingly the insoluble protein reserves formed approximately 89 % of the storage protein reserves in the seed and 96 % of these occurred in the haploid megagametophyte. Sodium dodecyl sulphate polyacrylamide gel electrophoresis indicated that the major storage proteins in radiata pine seed were glutelin-like that are comprised of 57-58 kDa subunits made up of 37-41 kDa and 21-23 kDa polypeptides linked by a disulphide bridge.

Storage protein reserves hydrolysis was observed in the embryo and megagametophyte during germination and early seedling growth. The megagametophyte storage proteins were metabolized and this resulted in a substantial increase in the free amino acid pool. However, there was very little increase in the

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List of Abbreviations

BSA	bovine serum albumin
DIC_{27}	days in culture at 27°C.
DAI_2	days of imbibition at 2°C.
DAI_{27}	days of imbibition at 27°C.
FW	fresh weight
G	gravity
H	hour
HCl	hydrochloric acid
kDa	kiloDalton
ME	mercaptoethanol
min	minute
ml	milliliter
mm	millimetre
mM	millimolar
PAGE	polyacrylamide gel electrophoresis
pI	isoelectric point
S	Sveldberg
SDS	sodium dodecyl sulfate
TAG	triacylglycerols
V	volt

1.0 Introduction

1.1 Radiata pine

1.1.1 Geography and economy

Pinus radiata (D. Don) is commonly known as radiata pine, Monterey pine and insignis pine. In this thesis *P. radiata* will be referred to as radiata pine. Critchfield *et al*, 1966 noted that radiata pine was the most widely planted pine in the world. Radiata pine is endemic to three distinct areas off central-coastal California at San Mateo, Monterey and San Luis Obispo Counties (Figure 1). Two other small isolated natural populations are found on Cedros and Guadalupe islands off of the west coast of Baja California, Mexico (McDonald and Laacke, 1990). The northernmost stand is east of point Año Nuevo in San Mateo County north of Santa Cruz at Swanton. The central stand is 48 km to the south near Monterey peninsula and Carmel in Monterey County, and the southernmost stand about 105 km away in the Pico Creek-Cambria area in San Luis Obispo County (Griffin *et al*, 1972). The north-south range is about 209 km and rarely is the pine found more than 11 km from the sea. The estimated total area occupied by natural stands of radiata pine on the United States mainland, range from 4860 to 6480 ha (Offord, 1964). The most abundant is found in the Carmel slopes next to Monterey. It is now difficult to determine the precise natural limits because of conspicuous amounts of new regeneration of radiata pine as additional trees have been planted that produced seed which led to many hectares of new reproduction. The southern part of the forest at Año Nuevo, for example, is estimated to have increased by as much as 95 ha in recent

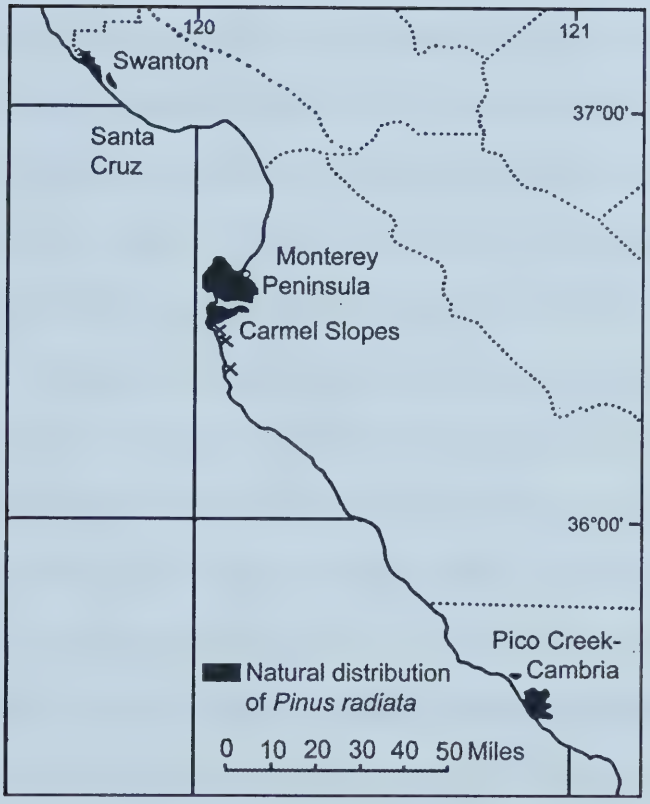


Figure 1. Natural distribution map of radiata pine (*Pinus radiata* D. DON.) in the United States. Shaded area indicates radiata pine native range.

decades (Griffin, 1981). Nevertheless, the total area currently occupied probably is no more than 8000 ha (Libby *et al*, 1968).

Though radiata pine is of little commercial importance in the United States as a timber species, it was the most widely planted pine tree in the world (Critchfield *et al*, 1966). Through the developmental tree plantations, the productivity of radiata pine has greatly increased, as has its economic importance. It was the leading introduced conifer species in Australia, New Zealand, and Spain (Scott, 1960) and a major conifer species in plantations of Argentina, Chile, Uruguay, Kenya, and the Republic of South Africa. In these countries the wood is utilized in the pulp and paper industry and also to manufacture insulation boards, fiberboard, plywood and veneers. The wood is also used in framing and construction and is an important source of tall oil. Tall oil is a dark, odorous liquid by-product of the sulfate process of paper manufacture. It is principally a mixture of resin acids, such as abietic acid, and fatty acids, such as oleic and linoleic acids, with some sterols and other compounds. After it is refined, tall oil is used to make coatings, sizing for paper, paint, varnish, linoleum, drying oils, emulsions, lubricants, and soaps. It is obtained by chemically treating the cooking liquor used in the operation of pulping wood for paper. In New Zealand and Australia radiata pine is often grown in shelterbelts in rural areas. The shelterbelts are small areas or strips of land in permanent vegetation, consisting of plantings of single or multiple rows of trees or shrubs that are designed to intercept pollutants, reduce wind erosion, protect growing plants, manage snow, improve irrigation efficiencies, provide wildlife habitat, and protection to livestock and structure (Agriculture and Agri-Food Canada, 2000). In many of these countries

where it has been introduced, radiata pine is a mainstay of the forest economy, serving internal markets, generating valuable foreign exchange reserves as an export, and reducing cutting pressure on native forests.

1.1.2 Mature seed morphology

The mature conifer seed has three main structural components; the seed coat, the megagametophyte and the embryo (Fig. 2). The seed coat is external to the megagametophyte and is formed from the combination of two outer integuments. Internal to the seed coat is a separate papery membrane formed from a third integument that covers the entire megagametophyte and acts as a barrier to water adsorption (Singh and Johri, 1972; Owens and Molder, 1977; Baron, 1978; Owens *et al*, 1982). In *P. sylvestris,* this has been described as a layer formed by the growth of nucellar cells into the endotesta (Tillman-Sutela and Kauppi, 1995). This conclusion was based on light microscopy observation of mature seeds and hence should be viewed with caution until a rigorous developmental study is carried out. Inside of the thin papery layer and covering the micropyle area of the megagametophyte, is a thin cap-like structure. This has been described as a remnant of the nucellus in gymnosperms (Singh and Johri, 1972).

In radiata pine, the diploid embryo consists of six to eight cotyledons and an embryonic axis that lies longitudinally within the megagametophyte separated by a space called the corrosion cavity (Singh and Johri, 1972; Hoff, 1987). The embryonic axis is composed of the shoot apical meristem, hypocotyl and radicle. The radicle is comprised of a subapical root meristem covered by an extensive root cap.

Figure 2. Light micrograph of long section through paraffin-mature radiata pine w
integuments removed. Megagametophyte (MEG) and embryonic axis (EMB). Ba
700 μm (Courtesy of Sandra Stone).


MEG
EMB
700 µm

The megagametophyte is a maternally derived haploid storage tissue that is formed prior to fertilization. The bulk of conifer seed storage reserves are found in this tissue (Ching, 1966; Owens *et al*, 1993). Smaller quantities of seed storage reserves are located in the embryo (Simola, 1974, Krasowski and Owens, 1993).

1.2 Storage proteins

The major storage reserves in conifer seeds are proteins and triacylglycerols (Misra, 1994). Since this thesis deals with seed storage proteins specifically, the nature of the TAG reserve in seeds will not be considered in this introduction.

Seed storage proteins are proteins that are accumulated during seed development, usually in organelles called protein bodies. They serve as a source of nitrogen for the developing seedling. Seed storage proteins make up at least 5% of the total protein in mature seed and are rapidly broken down following germination during early seedling growth (Derbyshire *et al*, 1976). They are classified into four groups based on their solubility; albumins, globulins, glutelins and prolamins (Osborne, 1918; Higgins, 1984). Albumins are soluble in aqueous buffers; whereas globulins are insoluble in aqueous buffers but soluble in salt solutions. Prolamins are only soluble in aqueous alcohol. Glutelins, originally classified by Osborne (1918) as insoluble in water, salt and alcoholic solutions but soluble in dilute acid or alkali, are more soluble in chaotropic agents such as sodium dodecyl sulfate (SDS) or urea (Koie and Nielson, 1977).

The classification of seed storage proteins is sometimes based on the ultracentrifugation sedimentation coefficient (S) of the holoprotein; for example,

globulins typically have an S coefficient of 11 and albumins commonly have an S value of 2 (Derbyshire *et al*, 1976; Shewry *et al*, 1995). Of the seed storage proteins, globulins are the most widely distributed group of storage proteins. They are present to varying degrees in the seeds of most angiosperms and gymnosperms (Shewry *et al*., 1995, Higgins, 1984).

The classification of major conifer storage proteins is quite confusing due to their shared similarity to other angiosperm seed storage proteins. Pinaceae seed storage proteins have been labelled crystalloids, legumins, legumin-like, globulin-like, glutelins and glutelin-like in several studies. The Pinaceae seed storage proteins were initially referred to as crystalloids, because their solubility properties were similar to the 11S crystalloid proteins of castor bean seeds (Gifford *et al*., 1982; Gifford, 1988), and both castor bean and Pinaceae seeds had protein vacuoles that contained a proteinaceous crystalloid (Stone and Gifford, 1997; Gifford *et al*., 1982). Given the solubility of the conifer storage proteins in chaotropic agents, these proteins will be referred to as glutelin-like in this thesis. Similar to the castor bean crystalloid proteins, the conifer 11S glutelin holoprotein consist of 6 identical subunits, each about 50 kDa (Gifford, 1988; Gifford and Bewley, 1983). Each subunit consists of a large (~30 kDa) and a small (~20 kDa) polypeptide linked by a disulphide bridge. In conifers, both large and small polypeptides are basic (Hakman *et al*., 1990; Allona *et al*., 1992), but in castor bean the large polypeptide is acidic and the small polypeptide is basic (Gifford and Bewley, 1983).

Glutelin-like 11S storage proteins have been described in *Pinus* species (Gifford 1988), *Picea* species (Gifford and Tolley, 1989; Misra and Greene, 1990;

Hakman, 1993), *Pseudotsuga mensiesii* (Douglas fir) (Green *et al.*, 1991), *Metasequoia glyptostroboides* (dawn redwood) (Hager and Dank, 1996), *Cedrus deodora*, *Larix laricana* (tamarak), and *Larix sibirica* (Siberian larch) (Jensen and Lixue, 1991). Glutelin-like storage proteins are absent in seeds of some conifer species, for example, *Abies* (Jensen and Lixue, 1991; Allona *et al*, 1994), *Cedrus atlantica* and in the Taxodiaceae (Allona *et al*, 1994). *Cedrus atlantica* seeds do contain a 55 kDa protein, which is composed of a 21 and 32 kDa polypeptides joined by a disulphide bridge. This protein is fully extracted in salt solutions and is likely a seed globulin (Allona *et al*, 1994). Gel filtration could not detect a larger holoprotein composed of salt extracted *Cedrus* 55 kDa subunits even though salt extractions usually allow the subunits of the holoprotein to remain associated. Interestingly, the 21 kDa polypeptide was recognized by a monospecific polyclonal antibody raised against the 21kDa glutelin-like polypeptide of *Pinus pinaster* (Allona *et al*, 1994). This same antibody also showed cross reactivity with the 20-22 kDa globulin polypeptides from pea and soybeans (Allona *et al.*, 1992). A salt soluble globulin has also been described in *Ginkgo biloba* of the Ginkgoaceae (Jensen and Berthold, 1989). This subunit forms a 11S hexameric holoprotein called ginnacin that is made up of 57 kDa subunits (Arahira and Fukazawa, 1994). The subunit is made up of two polypeptides. The partial deduced amino acid sequence from the 20 kDa peptide has 32-49 % homology to globulin seed storage proteins in both angiosperm monocots and dicots (Hager *et al.*, 1992; Hager *et al.*, 1995).

While the major pinaceae storage proteins can be referred to as glutelin-like because of their solubility in chaotropic agents, it should be noted that glutelin-like

storage proteins in monocotyledonous plants are a complex group of proteins that have a wide variety of subunit and holoprotein structures. In relation to this, rice seed glutelins are the most similar to the proteins of the Pinaceae. However, even though rice and Pinaceae glutelin-like genes have some sequence homology, monospecific polyclonal antibodies to the 21 kDa glutelin polypeptide from *Pinus pinaster* seeds did not recognize any proteins from rice glutelins (Allona *et al.*, 1992).

1.3 Seed Growth and Development

In pine, three phases of development occur between seed maturity and seedling autotrophy. These are seed dormancy breaking, germination and early seedling growth.

1:3:1 Seed dormancy

Seed dormancy occurs when viable seeds lose their capacity to germinate as a result of intrinsic physical or molecular blocks even when exposed to favorable environmental conditions (Bewley, 1997). When such physical and molecular blocks to completion of germination occurs before dispersal from the parent plant, the condition is referred to as primary dormancy. The hydrated seeds may acquire secondary or induced dormancy following seed dispersal, when exposed to certain environmental conditions. Secondary dormancy may be broken when the seeds are exposed to favorable environmental conditions, certain priming treatments such as

light, or a period of low or alternating temperature. Embryo germination may be inhibited in many cases by the seed coat and surrounding tissues through interference with absorption of water or gas exchange, mechanical restraint of the embryo, and possible supply of inhibitors to the embryo or prevention of chemicals from exiting the embryo (Bewley and Black, 1994). Dormancy in which germination is inhibited by the seed coat or by tissues surrounding the embryo while the embryo can germinate when isolated is referred to as coat-imposed dormancy (Bewley and Black, 1994). The embryo in some cases may be dormant due to factors inherent in the embryo itself, in which case the seed exhibits embryo dormancy. In tree species, embryo dormancy may be the result of a prematurely dispersed seed in which case germination can only occur after the seeds reach physiological maturity (Kozlowski and Pallardy, 1997). ABA has been shown to cause embryo dormancy in several species (Bewley, 1997). Varying degrees of coat imposed and embryo dormancy are exhibited by some tree species (Kozlowski and Pallardy, 1997). For example, dormant mature desiccated Pinaceae seeds often have a low rate of germination when imbibed at a given germination temperature (Schneider and Gifford, 1994). The embryos of mature Pinaceae seeds are non-dormant; they can germinate in the absence of all or some of the surrounding tissues (Kao and Rowan, 1970; Baron, 1978; Carpita *et al*, 1983; Downie and Bewley, 1996; Bianco *et al*, 1997; King 1998). Dormancy breaking is the phase of development during which the factors imposing dormancy are alleviated, allowing the seeds to germinate when placed in appropriate conditions. Imbibition at low temperature or alternating high and low temperatures over a period of time, called stratification, breaks dormancy in Pinaceae seeds.

Dormancy may be broken by chemicals, mechanical scarification, and heat and by after-ripening or storage of seeds in dry conditions (Kozlowski and Pallardy, 1997). In radiata pine, there was an increase in organic phosphate, organic acid, sucrose and high-energy phosphate levels during stratification (Kao and Rowen, 1970).

1.3.2 Germination

Germination begins after the seed is imbibed and placed at a suitable germination temperature. During the initial phase of water uptake existing cellular structures are hydrated and metabolism resumes (Obroucheva and Antipova, 1997). The biochemical events that take place during these first few hours are dependent upon organelles and enzymes that were present in the desiccated seed (Bewley and Black, 1994), and upon proteins synthesized from stored mRNAs (Beltran-Pena *et al.*, 1995). Within a few hours after the onset of imbibition, cells begin *de novo* synthesis of enzymes and cellular structures necessary for the processes involved in radicle emergence and early seedling growth (Obroucheva and Antipova, 1997). During germination the cells of the radicle grow primarily by expansion (Bewley and Black, 1994); cell division may or may not play a role in this growth. Radicle extension appears to be the result of turgor pressure exerted by cells of the radicle resulting from solute accumulation, possibly enhanced by breakdown of reserves within these cells. Cell expansion is aided by the loosening of the cell walls. Towards the end of germination, seed tissues constraining the tip of the radicle may also be weakened by enzymatic degradation, which aids radicle protrusion through the seed coat (Bewley,

1997). Germination is completed when the radicle emerges from the seed coat (Bewley, 1997). In conifers, germination is epigeal. Seedlings bear a whorl of five to nine cotyledons that are succeeded by primary needles. Secondary needles in fascicle bundles form when the seedling is a few months old. The root system of most conifer seedlings consists of a slender taproot, aimed straight down.

1.3.3 Early seedling growth

In conifers, early seedling growth refers to the developmental growth period from the time of radicle emergence to the shedding of megagametophyte from the seedling cotyledons. During this developmental phase, the seedling hypocotyl elongates and the seedling begins to differentiate to form the organs of the mature plant, leaving the cotyledons in contact with the megagametophyte (Gifford and Foster, 1989). It is during early seedling growth that most of the stored reserves are broken down and the products of this breakdown metabolized (Ching, 1966). These stored reserves, mainly, lipids, and protein in conifer seeds, supply the nutrients that drive the metabolic and biosynthetic pathways required to support seedling growth (Bewley and Black, 1994). The seedling uses these metabolites as energy, carbon, and nitrogen sources as it develops and becomes photosynthetically autonomous (Sasaki and Kozlowski, 1969; De Carli *et al*, 1987). In general pine seeds develop the capacity to photosynthesize soon after their emergence of the seedling from the seed coat (Kozlowski and Pallardy, 1997).

1.4 The present study

There is limited knowledge of the events that occur at the cellular level during conifer seed germination and the early growth of the seedling. This is particularly true for radiata pine where there is a need for more basic information on the storage reserve composition or the biochemical changes to the reserves during these critical stages of development. The purpose of this thesis is to expand our knowledge base in this area.

Radiata pine seeds, like most conifer seeds, exhibit coat-imposed dormancy and therefore require cold treatment for dormancy breaking. The first part of this thesis examined the effect of stratification on radiata pine seed germination and growth. I also determined radicle size classes for the developmental stages prior to completion of germination and early seedling growth.

During germination and early seedling growth, the seedling is dependent on the megagametophyte for nutrients for initial development, since the bulk of the storage reserves are stored in this tissue. Because of the proximity of the megagametophyte and the embryonic axis, there is a relay of signals and nutrient exchange between these two tissues. The second part of this thesis deals with the relationship between the seedling and megagametophyte.

Using biochemical techniques, I identified the major storage protein reserves in the seed megagametophyte and embryo and examined how these proteins were mobilized during germination and early seedling growth. I also examined the role of

the megagametophyte in providing the amino acid for the seedling during its early growth and development.

2.0 Materials and Methods

2.1 Chemicals and Equipment

Research grade chemicals were purchased from BDH (Toronto, ON, Canada), Bio-Rad Laboratories Ltd. (Mississauga, ON, Canada), Fisher Scientific (Nepean, ON) and Sigma Chemical Co. (St. Louis, MO, USA). Unless otherwise stated, distilled, deionized, Milli-Q water (resistance ≥ 18 megohms.cm) obtained from a Milli-Q filtration water system (Millipore Corporation, Bedford, MA, USA) was used for preparing all aqueous solutions.

The microfuge E and DU-65 spectrophotometer used in this research were manufactured by Beckman (Richmond, BC, Canada). Gel dryer, Mini-Protean II electrophoresis cell system and molecular weight markers for one-dimensional gel electrophoresis were purchased from Bio-Rad.

Kimpak was purchased from Seedburo Equipment (Chicago, Il, USA). Incubators used for stratification and germination was manufactured by Controlled Environments (Winnipeg, MB).

2.2 Seed material

2.2.1 Stratification, germination and early seedling growth

Mature, desiccated radiata pine seeds were received as a gift from Carter Holt Harvey Forests, Roturua, New Zealand and maintained at –20 °C. All seeds were surface-sterilized at room temperature according to Groome *et al* (1991) with modifications. The seeds were placed in cheese cloth bags under running tap water for 30 min, followed by immersion in 0.02 % (v/v) Tween 20 for 30 min with

shaking. The bag was then rinsed under running tap water for 30 min, and then shaken for 10 min in 1 % (w/v) sodium hypochlorite. Finally, the seeds were rinsed with milli-Q water to remove the bleach, removed from the cheesecloth bag with sterile tweezers, and transferred between layers of moist Kimpak in an autoclaved germination tray (all autoclaving was for 20 min at 1.3 kg/cm^2).

To determine the optimal stratification period for radiata pine seeds, batches of seeds were incubated at 2 °C and harvested at 7-day intervals. Harvested seeds were surface sterilized with 1 % sodium hypochlorite (w/v) for 10 min and rinsed with milli-Q water to remove the bleach, and placed on autoclaved moist kimpak in autoclaved germination trays and imbibed at 27 °C for 7 days in an illuminated incubator (19 $\mu mol\ m^2\ s^{-1}$). The number of germinants was determined. Germination was considered complete when the radicle had just penetrated the seed coat (Bewley and Black, 1994). All subsequent growth immediately following completion of germination is early seedling growth. The percentage germination was determined daily for three independent replicates of 100 seeds each.

2.2.2 Radicle size class determination

Radicle size classes were determined for fourteen developmental stages of seedling growth based on radicle length measurements. Radicle length was measured as the distance from the tip of the radicle to the point of emergence of the hypocotyl from the seed coat. Radicle length was measured daily and size classes determined as the non-overlapping parts of the standard deviations for fifty seeds over a period of 14 days.

2.2.3 Tissue collection for biochemical analysis

Harvested seed materials from different stages of development were separated into megagametophytes and embryo/seedlings, frozen in liquid nitrogen and stored at –75 °C before any biochemical analysis. In this thesis, embryo refers to the sporophyte in mature and stratified seeds. Seedling refers to the sporophyte at all stages of post-embryonic growth.

2.3 Biochemical analysis of storage reserves

2.3.1 Protein extraction and quantification

Protein was extracted from radiata pine megagametophyte and embryo/seedling according to the differential extraction procedure of Gifford *et al* (1982). Isolated plant tissues were homogenized in 1 ml 50 mM sodium phosphate buffer (pH 7.5) using an ice-cold mortar and pestle. The slurry was collected in a 1.5 mL eppendorf tube, vortexed and centrifuged at 14000 g for 20 min at 4 °C. Using a Pasteur pipette, the supernatant containing the buffer soluble proteins was collected and stored on ice for biochemical analysis or heated for 5 min at 95 °C with an equal volume of Laemmli buffer (62.5 mm Tris-HCL [pH 6.8], 2 % [w/v] SDS, and 10 % [v/v] glycerol) and stored at –20 °C for SDS-PAGE analysis. Cotton swabs were used to remove the residual lipid off the side of the tubes. The pellet was washed with 1ml 0.05 M sodium phosphate buffer and centrifuged at 14,000 g for 20 min at 4 °C. Finally, buffer insoluble protein in the pellet was solubilized in 1ml Laemmli buffer by heating to 95 °C for 5 min. The slurry was cooled and centrifuged at 14000

g for 20 min and the supernatant collected and stored at –20 °C for SDS-PAGE analysis and quantitative protein determination.

Seed parts from both tissues were homogenized in 4 mL 0.05 M sodium phosphate buffer and centrifuged at 14,000 g for 20 min at 4 °C. The supernatants were collected and assayed for total soluble protein. The pellets were resuspended in 4 mL Laemmli buffer, heated for 5 min at 95 °C and centrifuged at 14,000 g for 20 min at room temperature. The supernatants were collected and assayed for buffer insoluble proteins. Protein was quantified by the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) as the standard.

2.3.2 Ninhydrin Soluble Amino Acid Assay

Megagametophytes and embryos/seedlings were extracted according to method of Groome *et al* (1991) with modifications. Seed tissues were homogenized in 1 ml 50 mM $NaHPO_4$ using ice-cold mortar and pestle and the slurry collected and centrifuged at 14,000 g for 20 min at 4 °C. The supernatant was collected and the extract was boiled for 5 min at 95 °C to remove proteins. The boiled extract was then centrifuged at 14,000g for 20 min at room temperature. The supernatant was assayed for soluble amino acids using the ninhydrin method of Rosen (1950). A cyanide-acetate solution was made up fresh by diluting 1 mL of a 10 mM sodium cyanide solution in 49 mL of 2.65 mM sodium acetate. Equal volumes (0.5 mL) of the acetate-cyanide solution and a 3 % solution of ninhydrin in methyl cellosolve was added to 1 mL of diluted supernatant in a test tube; the mixture was boiled in a water bath for 15 min. Immediately following, 5 mL of 50 % diluted propan-2-ol (v/v) was

added to the boiled mixture then left to cool for 20-25 min in the dark. The absorbance of the resultant solution was read at 570 nm.

2.3.3 Gel electrophoresis

One dimension SDS-PAGE was carried out on 0.75 mm 12 % acrylamide slab gels at 200 V using a Mini Protean II dual slab cell system (Bio-Rad) as outlined by Groome *et al* (1991). A 4 % T acrylamide stacking gel was used for all gels. Protein extracts were diluted with an equal volume of Laemmli buffer and heated for 5 min at 95 °C. For electrophoresis under reducing conditions, Laemmli buffer containing β-mercaptoehthanol was used.

Following electrophoresis, protein profiles were visualized by staining with coomassie brilliant blue-R (Burk *et al*, 1993). Relative molecular masses of proteins were determined by the method of Weber and Osborne (1969). Molecular mass markers used included phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa) soybean trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa)(Bio-Rad). The gels were dried using a Bio-Rad model 543 gel dryer.

2.4 Tissue culture experiments

Embryos and megagametophytes from surface sterilized and fully stratified 28 DAI_2 seeds were excised and collected under sterile conditions in an Envirogard hood disinfected with 0.4 % roccal. The seed coat and nucellus were removed and a longitudinal incision made on the megagametophyte with an autoclaved razor blade

and the embryo removed using a sterile probe. Isolated seedlings were cultured on tissue culture media (Table 1.1 & 1.2, Becwar *et al.*, 1990) in petri dishes and later transferred to magenta GA-7 boxes under the same conditions. The MSG vitamin stock solution was filter-sterilized. The media components were mixed in their appropriate concentrations with 2 g/L gelrite and autoclaved for 20 min. The medium was allowed to cool to 45 °C and dispensed at 100 mL/magenta box and 25 ml/petri dish. Corresponding tissues from intact seeds, with all tissues external to the megagametophyte removed, and cultured under the same conditions were used as controls. Seed parts were harvested every 2 days, quick frozen in liquid nitrogen and stored at –80 °C prior to biochemical analysis. Radicle emergence for embryos cultured with intact megagametophytes occurred at 2 DAI_{27}. Amino acid and storage protein quantification experiments, storage protein extraction and determination was as previously described.

Table 1.1 Components of the tissue culture media

Components	Mass/volume	units	standard amount added (ml/l)	Final Conc. (mg/l)
MSG MACRO				
KNO_3	1	g	100	100
KCl	7.45	g	100	745
KH2PO4 (monobasic)	1.7	g	100	170
$MgSO_4.7H_2O$	3.7	g	100	370
$CaCl_2.2H_2O$	4.4	g	100	440
MSG MICRO				
$MnSO_4.H_2O$	1.69	g	10	16.9
$ZnSO_4.7H_20$	0.86	g	10	8.6
$CuSO_4.5H_20$ (1 mg/ml)	2.5	ml	10	0.025
KI	0.083	g	10	0.83
$CoCl_2.6H_2O$(1mg/ml)	2.5	ml	10	0.025
H_3BO_3	0.62	g	10	6.2
$Na_2Mo_4.2H_2O$	0.025	g	10	0.025
MSG IRON (FeEDTA)				
$FeSO_4.7H_2O$	2.784	g	10	27.84
Na_2EDTA	3.724	g	10	37.24
MSG VITAMIN				
Thiamine	0.01	g	100	
Pyridoxin	0.01	g	100	
Nicotinic Acid	0.05	g	100	

(Becwar *et al.*, 1990)

Table 1.2 Components of the tissue culture media

COMPONENTS	mass/volume	Units
MSG MACRO	100	ml
MSG MICRO	10	ml
MSG VITAMIN	1	ml
FeEDTA	10	ml
MYO-INOSITOL	0.1	g
NH_4NO_3	0.8	g
SUCROSE	30	g
NUCN ACTIVATED CARBON	5	g

(Becwar *et al.*, 1990)

3.0 Results

3.1 Seed stratification, germination and radicle growth

The seeds used in this study were not uniformly dormant. In fact only 55.7 % of mature radiata pine seeds were dormant and required stratification to break this dormancy (Fig. 3). Following the stratification treatment, the percentage of seeds that germinated increased from 44.3 ± 5.5 % to 98.7 ± 0.6 % when imbibed for 7 days at 27 °C (7 DAI_{27}). A 28 day stratification period was chosen as optimal for this study. Stratification for 28 days at 2 °C (28 DAI_2) also enhanced uniformity of seed germination and seedling growth. Following stratification for 28 days, radicle emergence was detected in a small percentage of seeds at 2 DAI_{27}. However, 4 DAI_{27} was required for more than 80 % of the seeds to complete germination (Fig. 4). Consequently, in this study 4 DAI_{27} was chosen to represent the average time for completion of seed germination at 27 °C. Changes in radicle length following germination were also determined in this study. Seeds were stratified for 28 days and then imbibed at 27 °C for up to 12 days. The relationship between mean radicle length and DAI_{27} is shown in Figure 5. Since there was considerable variation in radicle length for each day measured (data not shown), distinct radicle size classes for each day of imbibition following germination were chosen to reduce the variability in the harvested material. The radicle size classes used in this study were determined based on the 20 % of seeds that fall within the non-overlapping region of the standard deviations. They are shown in Figure 5.

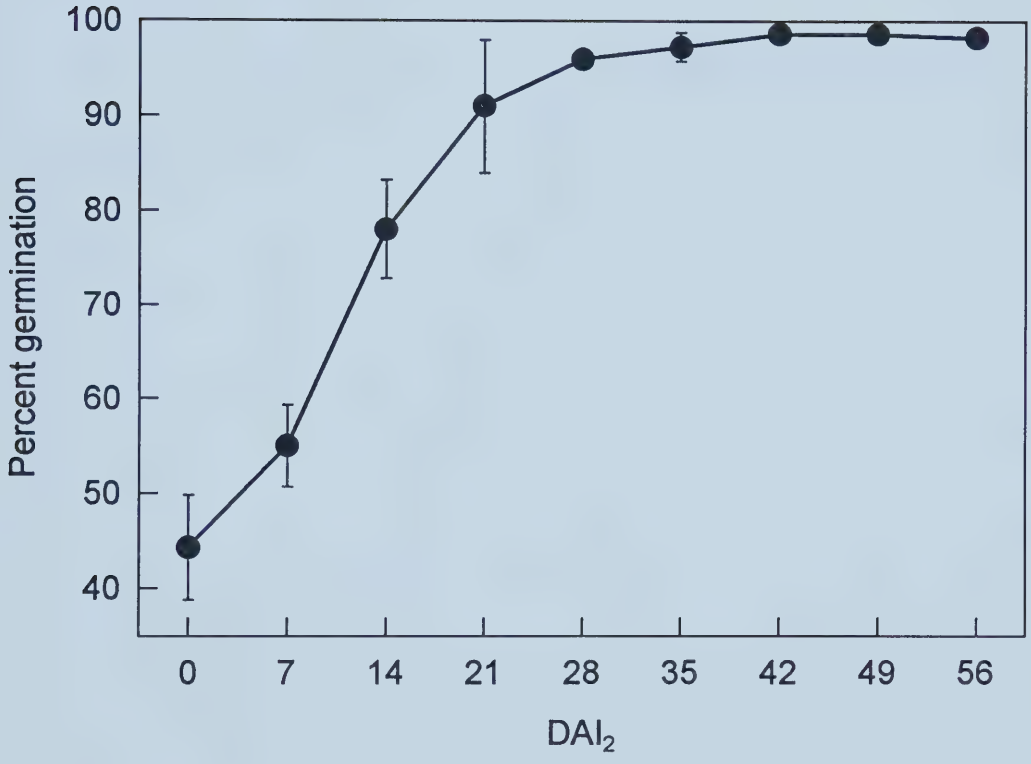


Figure 3. Changes in percent germination of seeds stratified at 2 °C for up to 56 days, harvested at 7-day intervals followed by imbibition at 27 °C for 7 days. Each data point is the mean of three replicates of 100 seed each ± standard deviation.

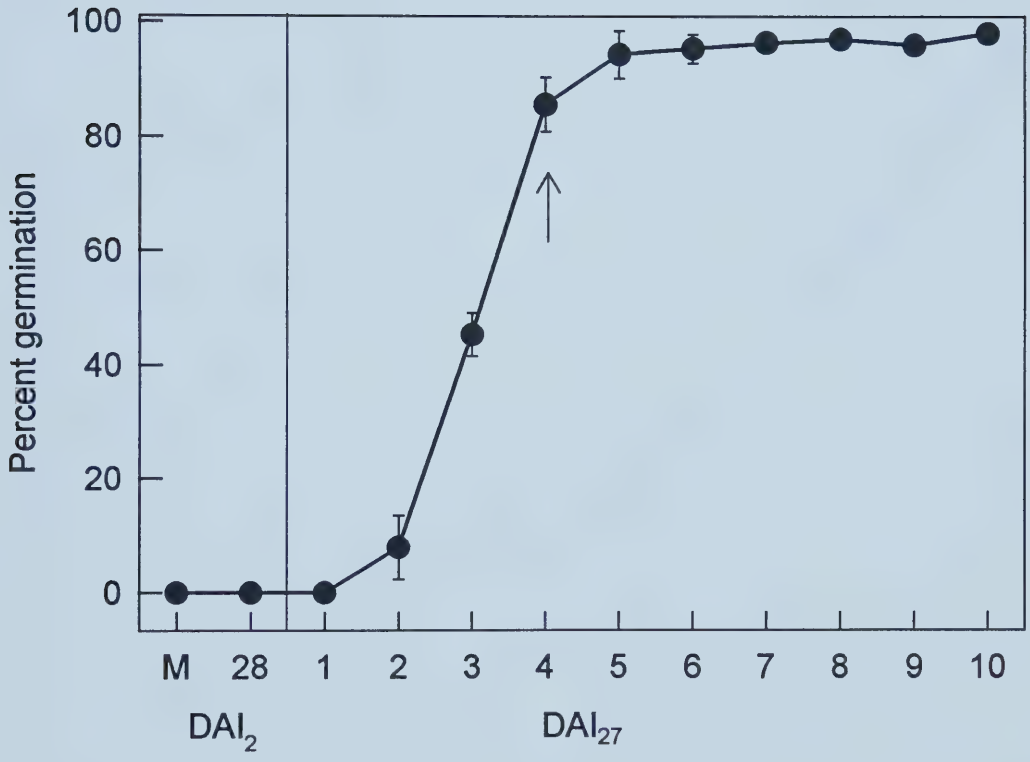


Figure 4. Changes in percentage germination of mature seeds and seeds stratified at 2 °C for 28 days followed by imbibition at 27 °C for 10 days. Each data point is the mean of three replicates of 100 seed each ± standard deviation. Arrow indicates the completion of germination by radicle emergence from the seed coat for > 80 % of seeds.

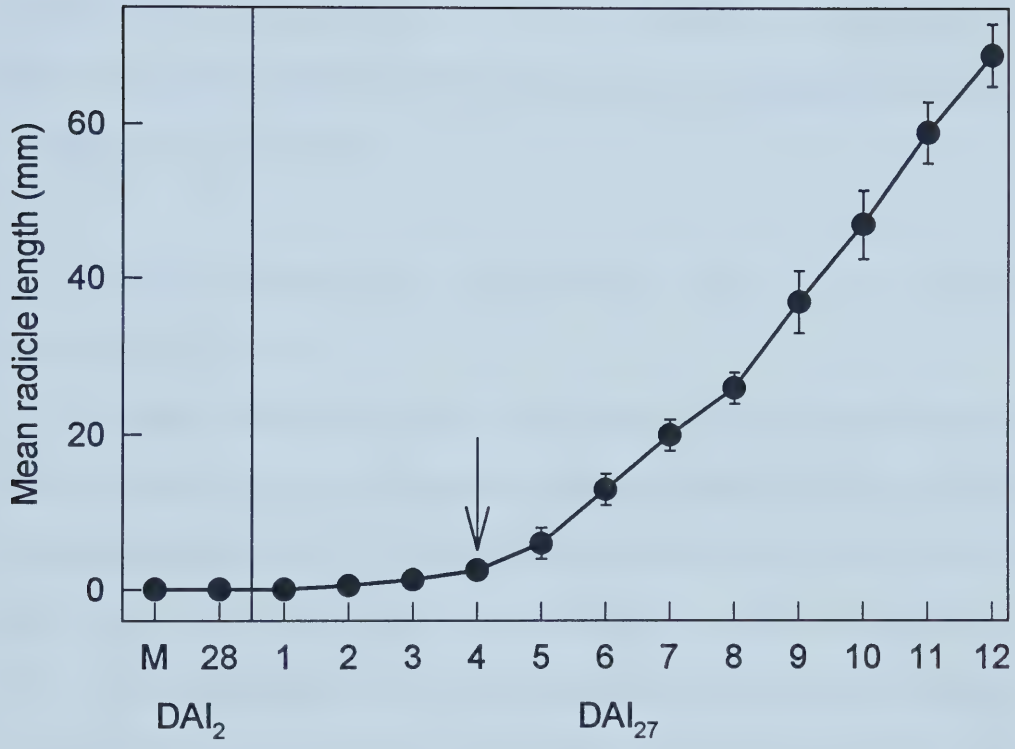


Figure 5. Changes in radicle lengths following seed imbibition at 27 °C and designated post-germinative radicle size classes. Each data point is the mean of 100 determinations. M, mature seed; 28, fully stratified seed; days of imbibition at 27 °C (DAI_{27}). Arrow indicates the completion of germination.

3.2 Quantification of seed storage protein in terms of seed fresh weight

The average total protein content of a mature radiata pine seed was 1.82 ± 0.09 mg (Table 2.0). Ninety three percent (1.68 ± 0.10 mg) of the total seed protein was in the haploid megagametophyte. Seed proteins were either phosphate buffer insoluble or phosphate buffer soluble. Eighty nine percent of the seed protein reserves in the megagametophyte and embryo were insoluble in phosphate buffer. The insoluble proteins accounted for 96 % of the protein in the megagametophyte and 46 % of the protein in the embryo.

3.2.1 Quantitative changes in the megagametophyte and embryo seed storage proteins following imbibition

The buffer soluble and insoluble protein reserves of the megagametophyte (Fig. 6) and embryo (Fig. 7) of mature radiata pine seed remained relatively constant throughout stratification at 2 °C. An initial hydrolysis (20 %) of the buffer insoluble protein reserves occurred by 2 DAI_{27}, immediately after the seeds were transferred to 27 °C. The insoluble protein reserve levels remained relatively constant until 5 DAI_{27} after which there was rapid depletion of the reserves. By 12 DAI_{27}, 94.2 % of the insoluble protein content of the megagametophyte had been depleted. In contrast, there was a 2.5 fold increase of the phosphate buffer soluble protein content of the megagametophyte by 10 DAI_{27} that decreased 1.5 fold by 12 DAI_{27}.

In the embryo, accumulation of the phosphate buffer insoluble proteins commenced after the seeds were transferred to 27 °C. There was a 27-fold increase

Table 2.0 Major seed storage proteins in mature radiata pine seed

Seed part	Insoluble (mg/seed part)	Soluble (mg/seed part)	Total (mg/seed part)
Megagametophyte	1.57 ± 0.09	0.12 ± 0.01	1.69 ± 0.10
Embryo	0.06 ± 0.01	0.07 ± 0.01	0.13 ± 0.01
Total (mg/seed part)	1.63 ± 0.09	0.19 ± 0.01	1.82 ± 0.09

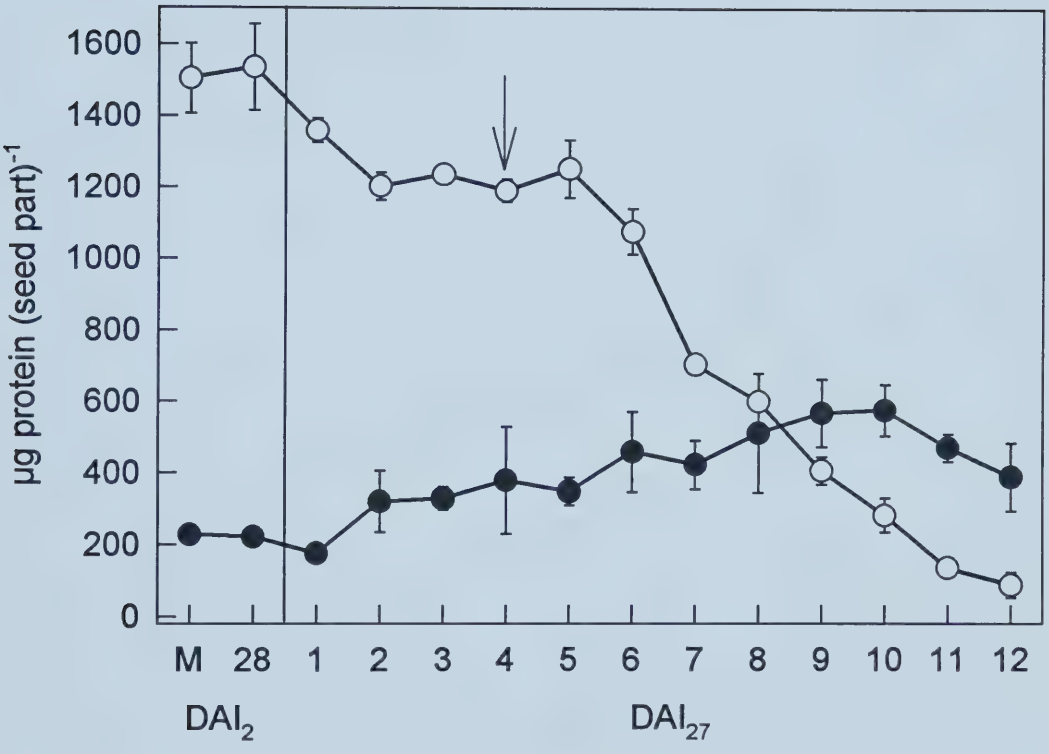


Figure 6. Quantitative changes in phosphate buffer soluble (●) and insoluble (○) proteins in the megagametophyte during germination and early seedling growth at 27 °C. X-axis shows mature seed (M), fully stratified seed (28 DAI_2), and seed imbibed for up to 12 days at 27 °C (1-12 DAI_{27}). Arrow indicates the completion of germination by radicle emergence from the seed coat. Each data point is the mean of three independent biological replicates each assayed in triplicate ± standard deviation of the mean.

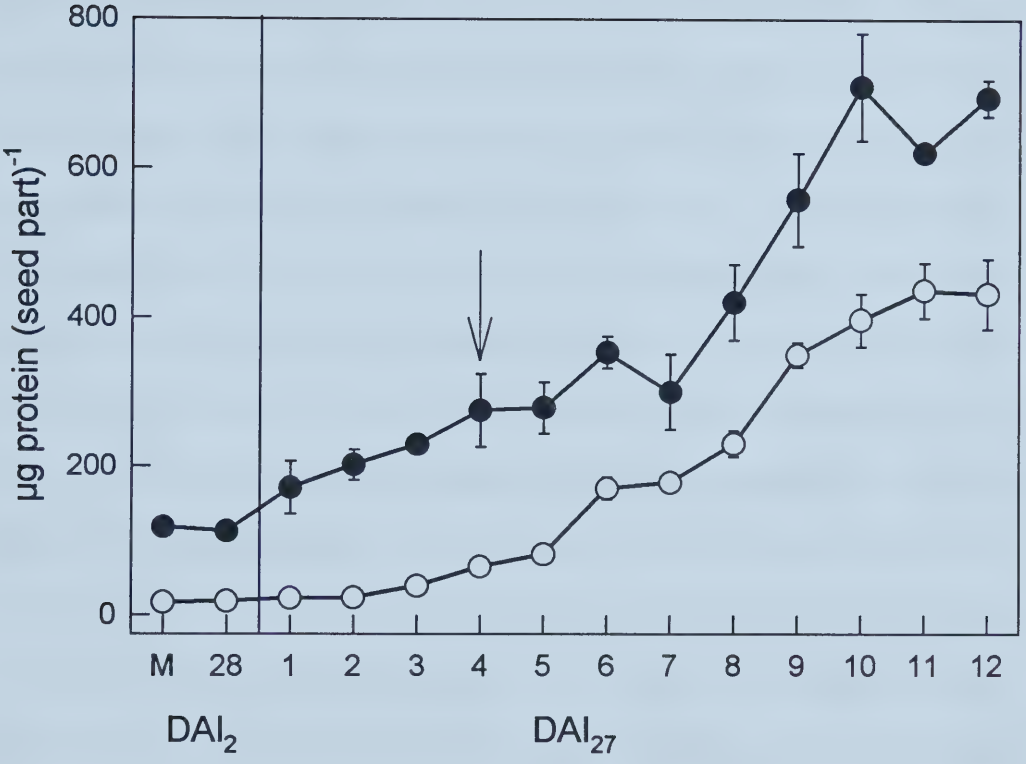


Figure 7. Quantitative changes in phosphate buffer soluble (•) and insoluble (○) proteins in the embryo/seedling during germination and early seedling growth at 27 °C. X-axis shows mature seed (M), fully stratified seed (28 DAI_2), and seed imbibed for up to 12 days at 27 °C (1-12 DAI_{27}). Arrow indicates the completion of germination by radicle emergence from the seed coat. Each data point is the mean of three independent biological replicates each assayed in triplicate ± standard deviation of the mean.

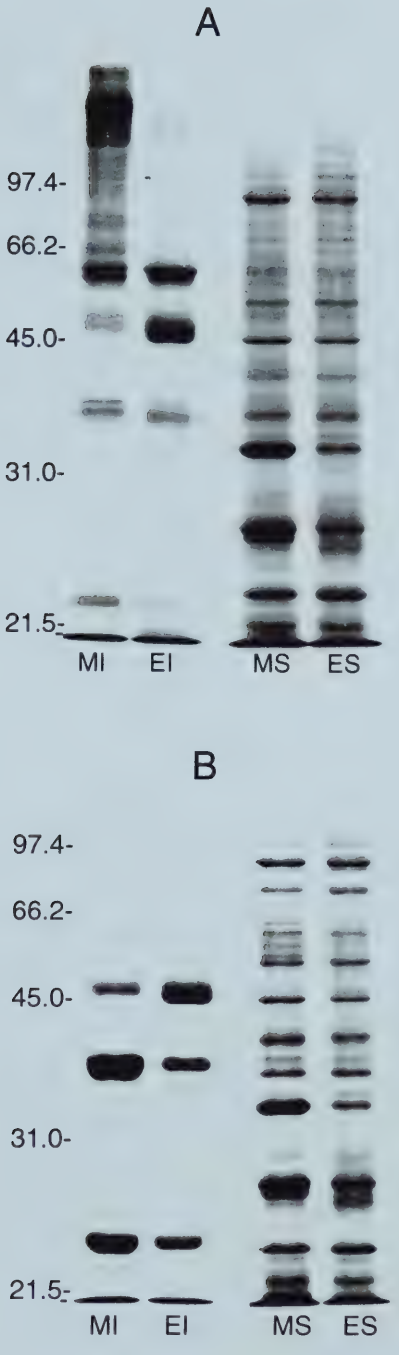
of the embryo insoluble proteins by 12 DAI_{27} and a 6-fold increase by 10 DAI_{27} of the buffer soluble protein content of the seedling.

3.3 Profiles of SDS dissociated proteins in mature seed

In order to determine the relative molecular masses of SDS dissociated proteins in the megagametophyte and embryo of mature radiata pine seeds, phosphate buffer soluble and insoluble proteins were separated and examined using SDS PAGE under reducing (+ME) and non-reducing (-ME) conditions. Under these conditions, the proteins from both tissues separated into several bands. The most noticeable bands from the profile of the megagametophyte under non-reducing conditions were two sets of proteins with molecular masses ranging from 57-58 kDa and 48-52 kDa (Fig. 8A, MI). Two other protein bands had molecular masses ranging from 38-39 kDa and 20-22 kDa. Equivalent proteins with solubility characteristics similar to those of the megagametophyte were also found in the phosphate buffer insoluble protein profile of the embryo under non-reducing conditions (Fig. 8A, EI). A group of proteins with very high molecular masses were observed in the megagametophyte and embryo but were present at very low relative intensity in the embryo insoluble protein profile under non-reducing conditions. In comparison, the 57-58 kDa and the 48-52 kDa polypeptide bands had higher relative intensities in the embryo than in the megagametophyte.

When reduced, the 57-58 kDa protein bands in both tissues disappeared and were replaced by two polypeptide bands with molecular masses of 37-41 kDa and 21-23 kDa (Fig. 8B, MI and EI). The 48-52 kDa polypeptide bands with solubility

Figure 8. Coomassie blue stained SDS-PAGE profile of phosphate buffer-soluble and -insoluble proteins from the megagametophyte and embryo of fully stratified seeds under (A) non-reducing and (B) reducing conditions. 3µg was loaded in 10 µl for insoluble proteins and 12.5 µg in 10 µl for soluble proteins. Relative molecular mass standards in kDa, shown by numerical values adjacent to gel profiles; MI, megagametophyte insoluble; MS, megagametophyte soluble; EI, embryo insoluble; embryo insoluble.


A
97.4-
66.2-
45.0-
31.0-
21.5-
MI
EI
MS
ES
B
97.4-
66.2-
45.0-
31.0-
21.5-
MI
EI
MS
ES

characteristics similar to the 57-58 kDa polypeptide was not affected by the mercaptoethanol treatment in either the megagametophyte or the embryo. All of the high molecular mass proteins disappeared when reduced.

In contrast to those obtained for the insoluble proteins, the reduced and non-reduced SDS dissociated phosphate buffer soluble protein profiles from the megagametophyte and the embryo of mature seed were similar, much complex and had many more bands (Fig. 8A and 8B, MS and ES). The most prominent polypeptide bands in the soluble profiles of the megagametophyte and embryo had molecular masses of 73 kDa, 47 kDa, 34.5 kDa, 27.5 kDa, 22 kDa and 20 kDa. Besides similarities, a specific difference was that the 34.5 kDa and 27.5 kDa polypeptide bands had relatively higher intensity in the megagametophyte than in the embryo. When reduced, the soluble proteins from both tissues remained not affected by the mercaptoethanol treatment.

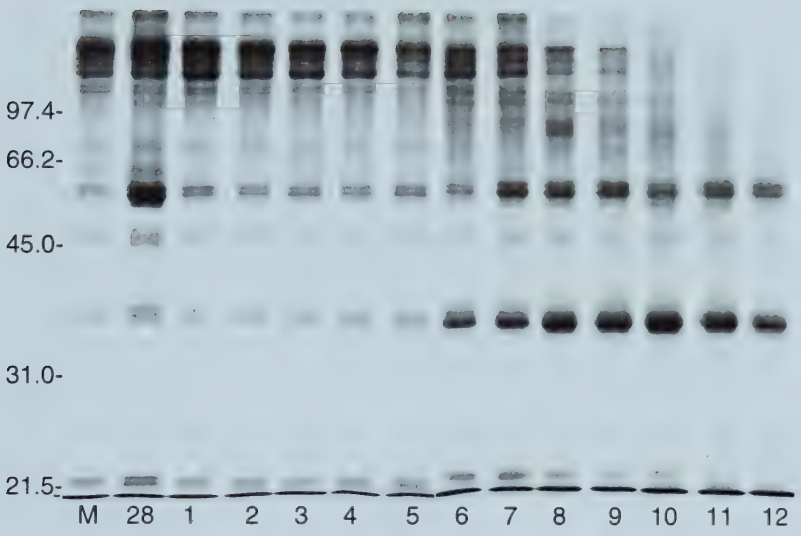
3.3.1 Qualitative changes in SDS dissociated proteins

To determine the storage function of the soluble and insoluble protein reserves of the megagametophyte and embryo/seedling, SDS PAGE protein profiles were examined during germination and early seedling growth. Storage protein hydrolysis was represented by a decrease in the relative intensity of the polypeptide bands.

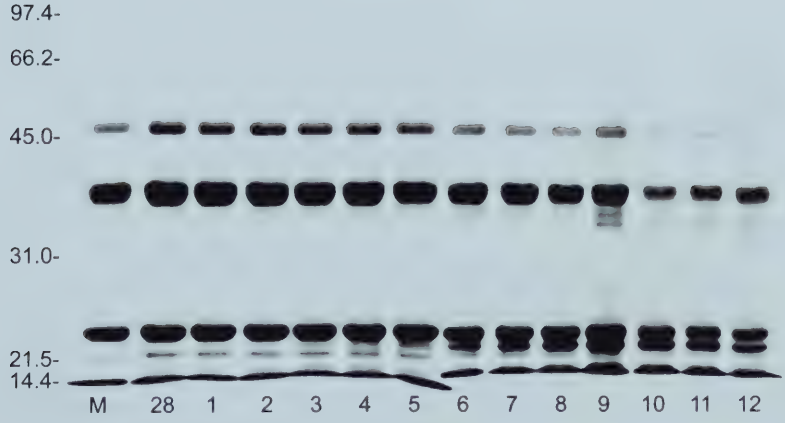
Under non-reducing conditions, the megagametophyte insoluble protein profile showed rapid hydrolysis of the 48-52 kDa, 38-39 kDa and 20-22 kDa protein bands by 12 DAI_{27} (Fig. 9A). Complete hydrolysis of the high molecular mass proteins was also observed after 9 DAI_{27}. The relative intensity of a 36.5 kDa polypeptide of the

Figure 9. Coomassie blue stained SDS-PAGE profile of phosphate buffer-insoluble proteins from the megagametophyte following imbibition under (A) non-reducing and (B) reducing conditions. For each lane 2.5 µg was loaded in 10 µl for insoluble proteins. Relative molecular masses of standards in kDa, shown by numerical values adjacent to gel profiles; M, mature seed; 28 DAI_2 seed, and seed imbibed up to 12 DAI_{27}.

A


97.4-
66.2-
45.0-
31.0-
21.5-
M 28 1 2 3 4 5 6 7 8 9 10 11 12

B


97.4-
66.2-
45.0-
31.0-
21.5-
14.4-
M 28 1 2 3 4 5 6 7 8 9 10 11 12

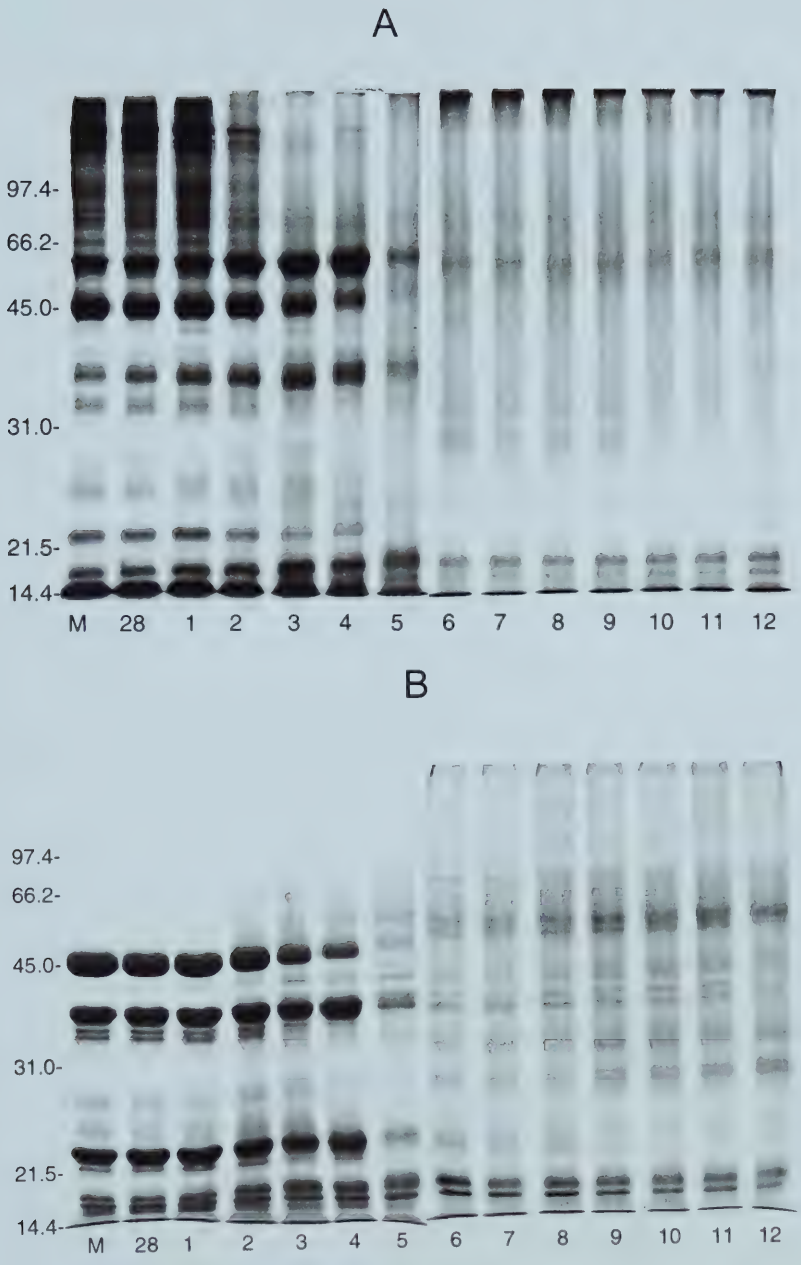
non-reduced insoluble fraction from the megagametophyte increased after 5 DAI_{27}. Most of the changes that occurred in the non-reduced seedling insoluble protein profile were very similar to that in the megagametophyte even though the equivalent polypeptides of the seedling under non-reducing conditions disappeared at a faster rate than in the megagametophyte.

By 5 DAI_{27}, the 57-58 kDa, 48-52 kDa and 21-22 kDa polypeptides had completely disappeared from the seedling (Fig. 10A). However, the 36.5 kDa polypeptide was absent in the non-reduced insoluble profile of the seedling.

The same pattern of protein hydrolysis was observed in the 48-52 kDa polypeptide from both tissues under reducing (Fig. 9B and 10B) and non-reducing conditions (Fig. 9A and 10A). In the megagametophyte, the hydrolysis of the 37-41 kDa and the 21-23 kDa polypeptides of the reduced gel profile occurred at a much slower rate but mirrored the rate of disappearance of the 57-58 kDa polypeptide bands of non-reduced gel profile. There was noticeable decrease in the relative intensities of these bands after 9 DAI_{27}. A 20 kDa polypeptide disappeared at a faster rate and had completely disappeared by 9 DAI_{27}. The 36.5 kDa polypeptide that accumulated under non-reducing conditions was absent in the reduced profile and was replaced by the 20.8 kDa after 4 DAI_{27} which increased in relative intensity similar to the 36.5 kDa polypeptide (Fig. 9B).

In the seedling, changes in relative intensity of the 48-52 kDa and 21-23 kDa polypeptides under reducing conditions showed marked decrease in relative intensity of their bands by 5 DAI_{27}, similar to their hydrolysis under non-reducing conditions (Fig. 10B). When reduced the two polypeptides that replaced the 57-58 kDa

Figure 10. Coomassie blue stained SDS-PAGE profile of phosphate buffer-insoluble proteins from the embryo/seedling following imbibition under (A) non-reducing (B) reducing conditions. For each lane 2.5 μg was loaded in 10 μl for insoluble proteins. Relative molecular masses of standards in kDa, shown by numerical values adjacent to gel profiles; M, mature seed; 28 DAI_2 seed, and seed imbibed up to 12 DAI $_{27}$.


A
97.4-
66.2-
45.0-
31.0-
21.5-
14.4-
M 28 1 2 3 4 5 6 7 8 9 10 11 12
B
97.4-
66.2-
45.0-
31.0-
21.5-
14.4-
M 28 1 2 3 4 5 6 7 8 9 10 11 12

polypeptides, showed a similar pattern of protein hydrolysis compared to the larger heterodimer. One major difference between the megagametophyte and embryo was the absence of the 20.8 kDa polypeptide that appeared at 4 DAI_{27} and increased thereafter.

3.3.2 Qualitative changes in the seedling soluble proteins during germination and early seedling growth

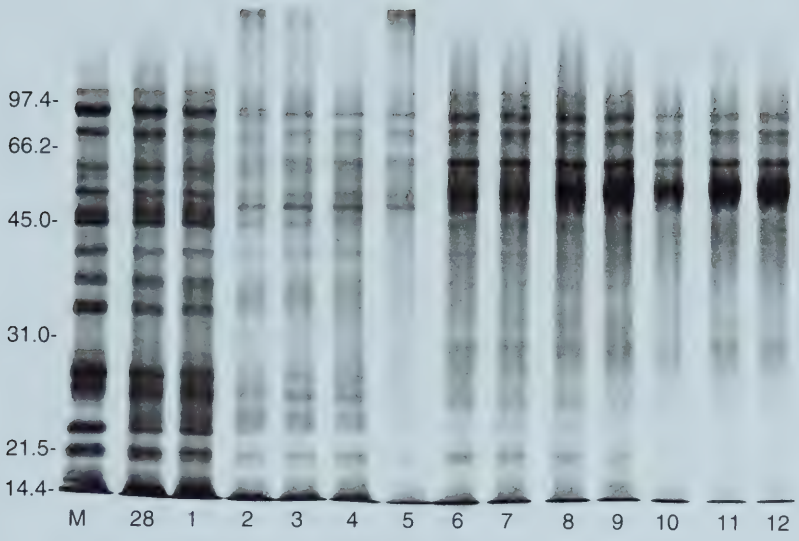
The pattern of protein hydrolysis of the buffer soluble SDS dissociated proteins under non-reducing and reducing conditions was very similar in both tissues during germination and early seedling growth. Fig. 11A and 11B show buffer soluble protein profile obtained from the seedlings of the all the developmental stages examined. The most noticeable change was the increase in relative intensity of a 53-55 kDa protein during germination and early seedling growth. Rapid decrease in the relative intensity of the 34.5 kDa, 73 kDa and 27.5 kDa polypeptides occurred at 2 DAI_{27}. A decrease in relative intensity of the 20 and 22 kDa polypeptides was also observed at the completion of germination, however their disappearance was more gradual thereafter.

3.3.3 Qualitative changes in the megagametophyte soluble proteins during germination and early seedling growth

In contrast to the seedling soluble proteins, there were noticeable changes between the megagametophyte non-reduced (Fig. 12A) and reduced (Fig. 12B) profiles. Under non-reducing conditions, the hydrolysis of the 22 and 27.5 kDa was

Figure 11. Coomassie blue stained SDS-PAGE profile of phosphate buffer-soluble proteins from the embryo/seedling following imbibition under (A) non-reducing and (B) reducing conditions. For each lane 10 µg was loaded in 10 µl for insoluble proteins. Relative molecular masses of standards in kDa, shown by numerical values adjacent to gel profiles; M, mature seed; 28 DAI_2 seed, and seed imbibed up to 12 DAI $_{27}$.

A


97.4-
66.2-
45.0-
31.0-
21.5-
14.4-
M 28 1 2 3 4 5 6 7 8 9 10 11 12

B

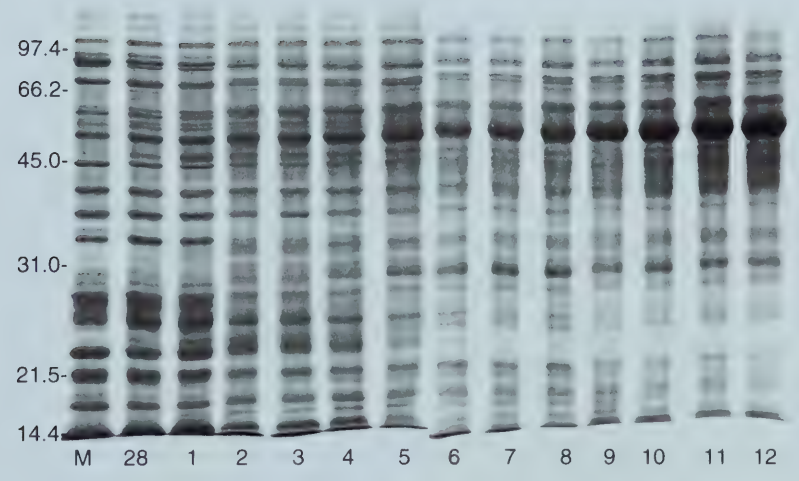
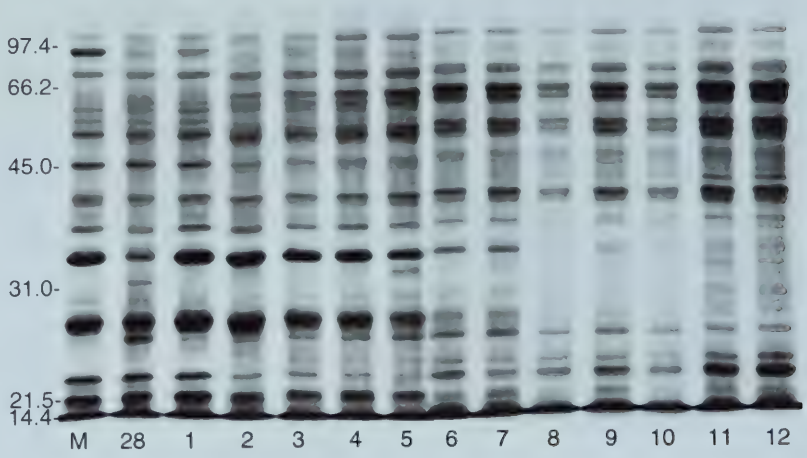
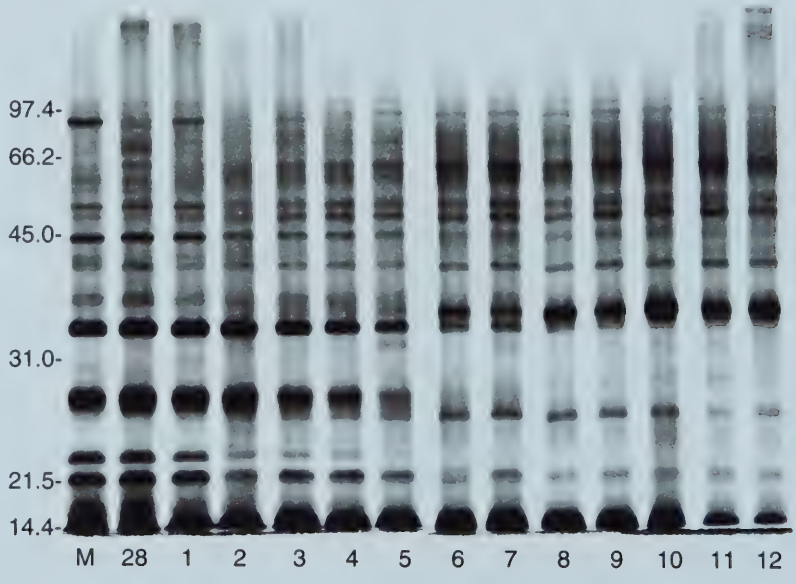

97.4-
66.2-
45.0-
31.0-
21.5-
14.4
M 28 1 2 3 4 5 6 7 8 9 10 11 12

Figure 12. Coomassie blue stained SDS-PAGE profile of phosphate buffer-soluble proteins from the megagametophyte following imbibition under (A) non-reducing (B) reducing conditions. For each lane 10 µg was loaded in 10 µl for insoluble proteins. Relative molecular masses of standards in kDa, shown by numerical values adjacent to gel profiles; M, mature seed; 28 DAI_2 seed, and seed imbibed up to 12 DAI $_{27}$.

A


97.4-
66.2-
45.0-
31.0-
21.5-
14.4-
M 28 1 2 3 4 5 6 7 8 9 10 11 12

B


97.4-
66.2-
45.0-
31.0-
21.5-
14.4-
M 28 1 2 3 4 5 6 7 8 9 10 11 12

complete by 5 DAI_{27}. The disappearance of the 34.5 kDa protein was quite noticeable after 4 DAI_{27} and was completely hydrolyzed by 9 DAI_{27}. The hydrolysis of the 20 and 47 kDa polypeptides occurred at a much slower rate. Interestingly there was the accumulation of two sets of proteins, a 25 kDa protein which started to accumulate at 3 DAI_{27} and a 34.5 kDa protein that started to accumulate after 5 DAI_{27}.

When reduced, the changes in the profiles of the 47 kDa, 34.5 kDa, 27.5 kDa, 22 kDa and 20 kDa proteins were similar to the non-reduced gel protein profile. There was the accumulation of a 61 kDa, 55 kDa, 41.5 kDa and 25 kDa polypeptides. The most noticeable change in the non-reduced protein profile is the rapid hydrolysis of the 34.5 kDa band by 9 DAI_{27}. The 22 kDa polypeptide was completely depleted by 5 DAI_{27} and more noticeably by 3 DAI_{27}. The 27 kDa band was also depleted by 5 DAI_{27} and another band 26 kDa started to accumulate by 3 DAI_{27}.

3.4 Quantitative changes in soluble amino acid content in the megagametophytes and seedlings

The soluble amino acid pool in both seedlings and megagametophyte was relatively constant during germination (Fig. 13). Following germination, however, the embryo soluble amino acid content showed a 300-fold increase by 12 DAI_{27}. In contrast the corresponding amino acid pool in the megagametophyte rose slightly by 9 DAI_{27} and then declined.

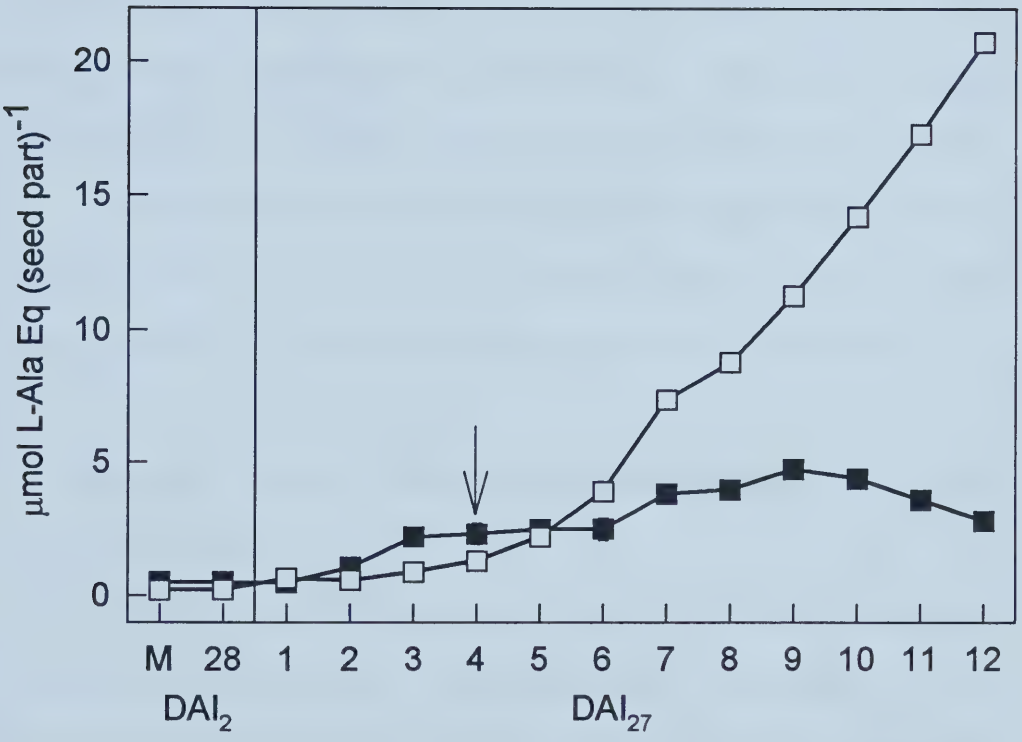


Figure 13. Quantitative changes in the soluble amino acid content of the seedlings (□) and megagametophytes (■) during germination and early seedling growth of seeds imbibed at 27 °C. X-axis shows mature seed (M), fully stratified seed (28 DAI_2), and seed imbibed for up to 12 days at 27 °C. Arrow indicates the completion of germination by radicle emergence from the seed coat. Each data point is the mean of three independent biological replicates each assayed in triplicate ± standard deviation of the mean.

3.5.1 Quantitative changes in the megagametophytes soluble and insoluble protein from *in vitro* cultured decoated seeds

The pattern of protein hydrolysis in the megagametophytes from cultured seeds with the coat and nucellus removed (Fig. 14) was similar to that of the intact seed grown *in vivo* (Fig. 6). There was a 20 % decrease in megagametophyte buffer insoluble protein during germination (Fig. 14). The insoluble protein remained relatively constant after completion of germination (2 DIC_{27}) and was rapidly depleted after 6 DIC_{27}. By 8 DIC_{27}, 75 % of the insoluble protein had been depleted.

In contrast, the buffer soluble protein of the megagametophyte remained relatively constant until the completion of germination when there was a two-fold increase by 8 DIC_{27}. The soluble proteins then decreased four fold by 12 DIC_{27}.

3.5.2 Quantitative changes in seedling soluble and insoluble proteins from *in vitro* cultured decoated seeds

The seedling cultured with associated megagametophyte intact also exhibited similar pattern to seedling soluble and insoluble protein accumulation of *in vivo* grown seeds (Fig. 15). There was no significant difference in the seedling insoluble protein content until after 4 DIC_{27} when it increased significantly (18 fold by 10 DAI_{27}). The seedlings insoluble protein on the other hand increased rapidly after 6 DIC_{27} by 7.5 fold.

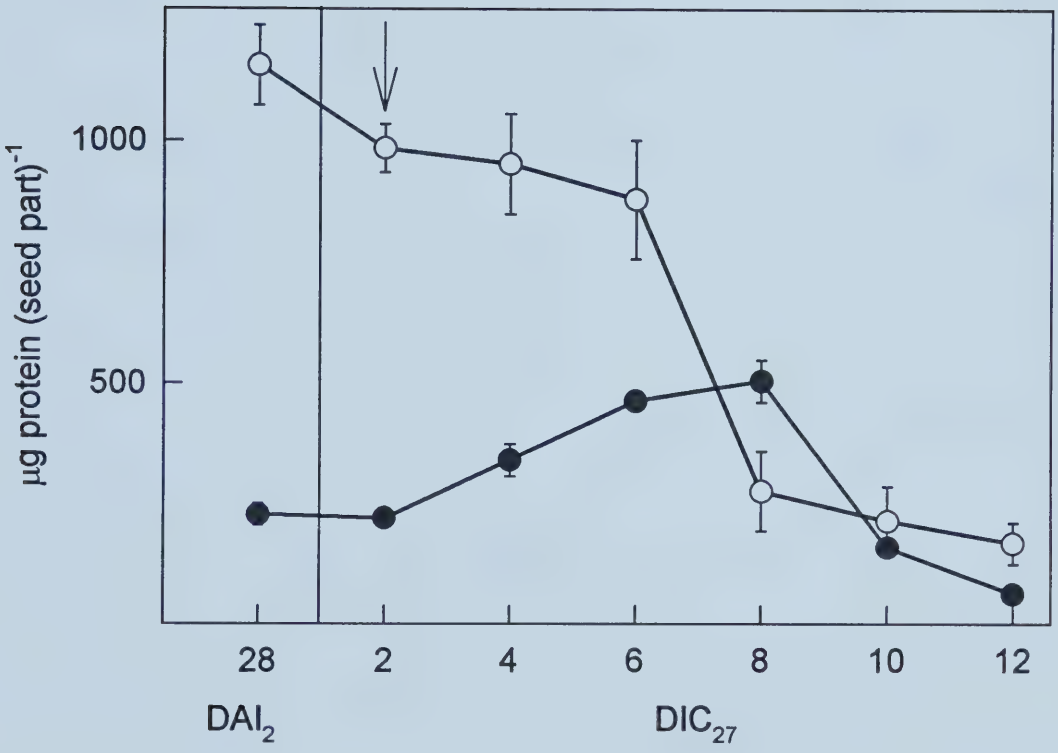


Figure 14. Quantitative changes in phosphate buffer soluble (•) and insoluble (○) proteins of *in vitro* cultured megagametophytes with intact seedlings during germination and early seedling growth of seeds imbibed at 27 °C. X-axis shows fully stratified seed (28 DAI_2), and seed imbibed for up to 12 days at 27 °C. Arrow indicates the completion of germination by radicle emergence from the seed coat. Each data point is the mean of three independent biological replicates each assayed in triplicate ± standard deviation of the mean.

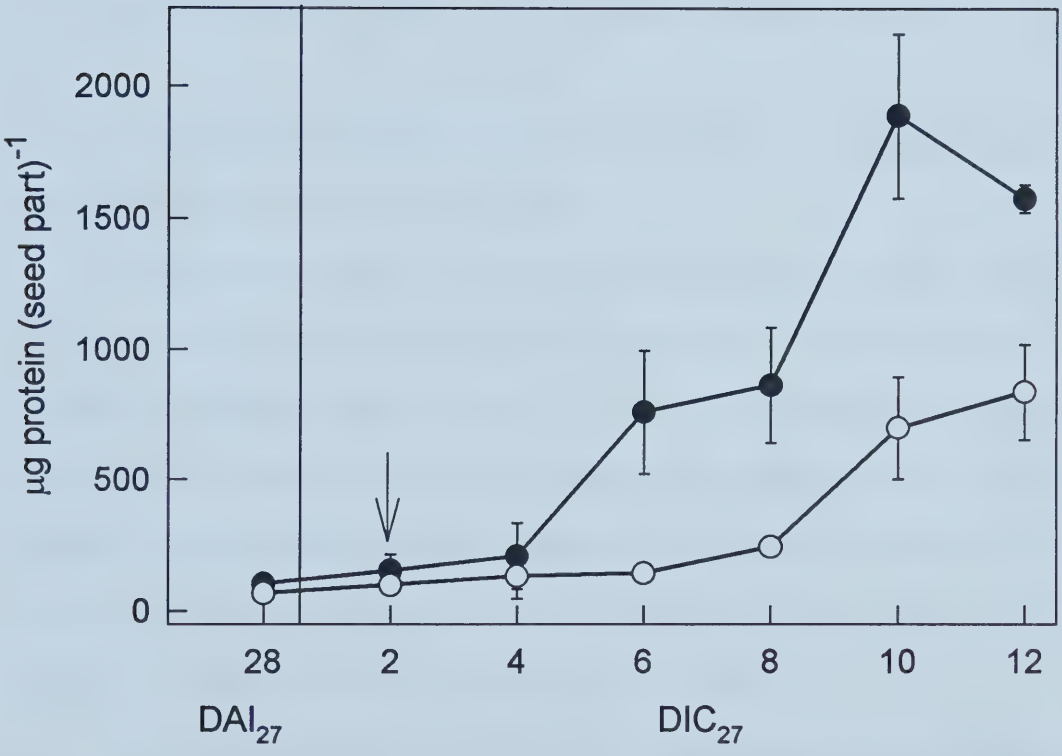


Figure 15. Quantitative changes in phosphate buffer soluble (○) and insoluble (●) proteins of *in vitro* cultured seedlings with intact megagametophytes during germination and early seedling growth of seeds imbibed at 27 °C. X-axis shows fully stratified seed (28 DAI_2), and seed imbibed for up to 12 days at 27 °C. Arrow indicates the completion of germination by radicle emergence from the seed coat. Each data point is the mean of three independent biological replicates each assayed in triplicate ± standard deviation of the mean.

3.5.3 Quantitative changes in soluble and insoluble proteins of *in vitro* cultured isolated seedling

In contrast, there was no significant difference in the soluble protein levels from cultured isolated seedlings of the different developmental stages (Fig. 16) throughout the study period. The insoluble protein levels increased after germination was complete at 2 DIC_{27} and peaked at 8 DIC_{27} then remained relatively constant.

3.6 Qualitative changes in SDS dissociated protein profile of the megagametophyte and seedling of *in vitro* cultured decoated seeds

To determine the storage function of the soluble and insoluble protein reserves of the megagametophyte and embryo/seedling, protein profiles were examined during germination and early seedling growth. The storage protein hydrolysis was represented by decrease in the relative intensity of the polypeptide bands. The patterns of protein hydrolysis under reducing and non-reducing conditions in the megagametophyte and seedling profiles from cultured seeds were similar to that grown *in vivo* (Fig. 9A, 9B, 10A, 10B, 11A, 11B, 12A, 12B).

In the megagametophyte, the 48-52 kDa storage proteins under non-reducing conditions (Fig. 17 A) decreased very slowly up to 8 DIC_{27} after which it increased again. The 20-22 kDa polypeptides of the megagametophyte did not disappear completely, although the relative intensity of this band decreased significantly by 12 DIC_{27}. When reduced (Fig. 17 B) the pattern of hydrolysis of the megagametophyte 48-52 kDa and 20-22 kDa polypeptides remained unchanged (Fig. 17 B). The high molecular mass proteins also diminished significantly after 6 DIC_{27}. There was

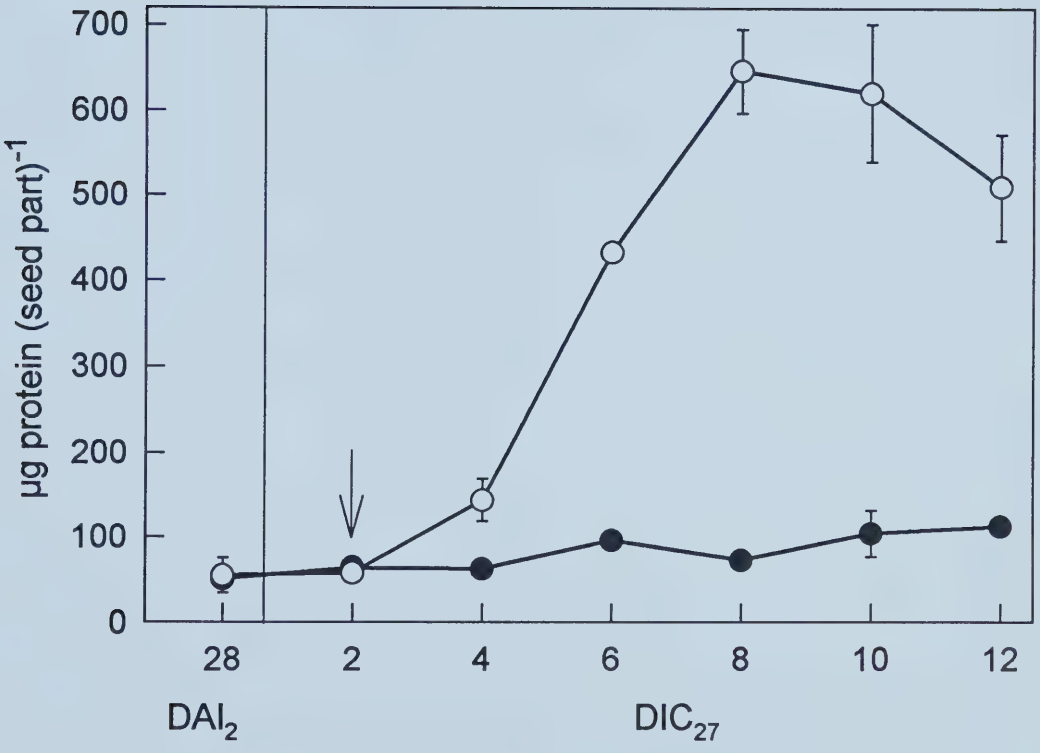
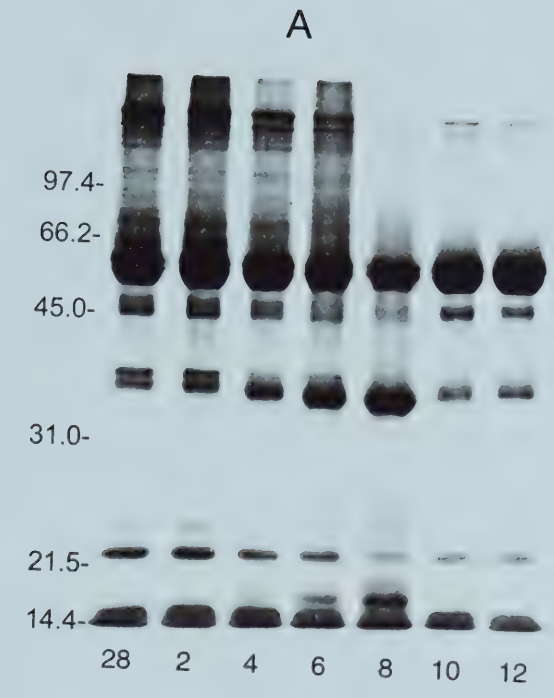


Figure 16. Quantitative changes in phosphate buffer soluble (•) and insoluble (○) proteins in isolated *in vitro* cultured seedlings during germination and early seedling growth at 27 °C. X-axis shows fully stratified seed (28 DAI_2), and seed imbibed for up to 12 days at 27 °C. Arrow indicates the completion of germination by radicle emergence from the seed coat. Each data point is the mean of three independent biological replicates each assayed in triplicate ± standard deviation of the mean.

Figure 17. Coomassie blue stained SDS-PAGE profile of phosphate buffer-insoluble proteins from the megagametophyte of *in vitro* cultured decoated seed with intact seedling under (A) non-reducing and (B) reducing conditions. For each lane 3 μg was loaded in 10 μl for insoluble proteins. Relative molecular masses of standards in kDa, shown by numerical values adjacent to gel profiles; 28 DAI_2 seed, and seed imbibed up to 12 DAI $_{27}$.


A
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97.4-
66.2-
45.0-
31.0-
21.5-
14.4-
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significant increase in relative intensity of the 36.5 kDa polypeptide observed under non-reducing conditions by 8 DIC_{27} after which it started to disappear. A 17 kDa polypeptide started to increase in relative intensity after 4 DIC_{27} and disappeared after 8 DIC_{27}.

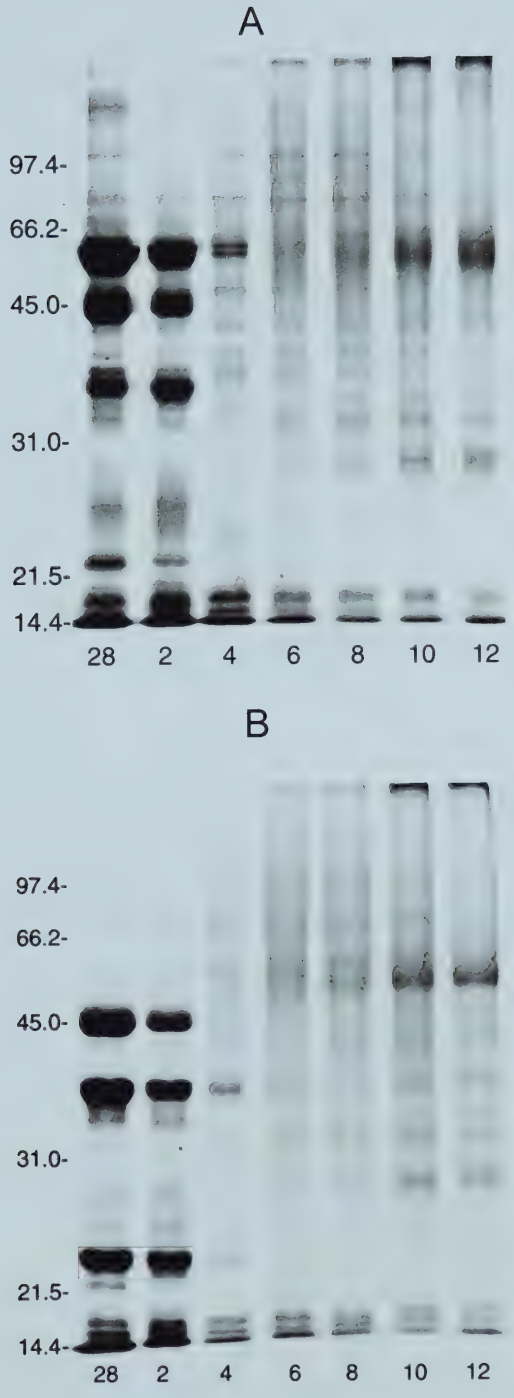
The changes that occurred in the non-reduced seedling insoluble protein profile (Fig. 18 A) were quite similar to that in the megagametophyte even though the equivalent polypeptides of the seedling under non-reducing conditions disappeared at a faster rate and most of the major storage proteins had disappeared by 4 DIC_{27}. Under non-reducing conditions, the 48-52, 38-39, 20-22 kDa polypeptides completely disappeared from the seedling after 2 DIC_{27}, (Fig. 18 A). The 57-58 kDa polypeptide disappeared completely after 4 DIC_{27}. There was however no accumulation of the 36.5 kDa polypeptide in the non-reduced insoluble profile of the seedling.

When reduced, the rate of disappearance of the seedling 48-52 kDa polypeptide (Fig.18 B) remained unaffected in both tissues.

In the megagametophyte, the hydrolysis of the 37-41 kDa and the 21-23 kDa polypeptides occurred at a much slower rate but mirrored the rate of disappearance of the 57-58 kDa bands. The 36.5 kDa polypeptide that accumulated under non-reducing conditions was absent in the reduced profile and was replaced by the 20.8 kDa which increased in relative intensity by 8 DIC_{27} similar to the 36.5 kDa polypeptide and decreased thereafter (Fig. 17 B).

In the seedling, changes in relative intensity of the 48-52 kDa polypeptide when reduced conditions showed marked decrease in relative intensity of their bands by 2 DIC_{27}, similar to their hydrolysis when not reduced (Fig. 18 A). When reduced

Figure 18. Coomassie blue stained SDS-PAGE profile of phosphate buffer-insoluble proteins from the embryo/seedling of *in vitro* cultured decoated seed grown with intact megagametophyte under (A) non-reducing and (B) reducing conditions. For each lane 3 µg was loaded in 10 µl for insoluble proteins. Relative molecular masses of standards in kDa, shown by numerical values adjacent to gel profiles; 28 DAI_2 seed, and seed imbibed up to 12 DAI $_{27}$.


A
97.4-
66.2-
45.0-
31.0-
21.5-
14.4-
28
2
4
6
8
10
12
B
97.4-
66.2-
45.0-
31.0-
21.5-
14.4-
28
2
4
6
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12

(Fig. 18 B) the 38-39 kDa and 20-22 kDa polypeptides showed a similar pattern of protein hydrolysis to the 57-58 kDa polypeptides and completely disappeared by 4 DIC_{27}. One major difference between the megagametophyte and embryo was the absence of the 20.8 kDa polypeptide that appeared at 4 DIC_{27} and increased thereafter.

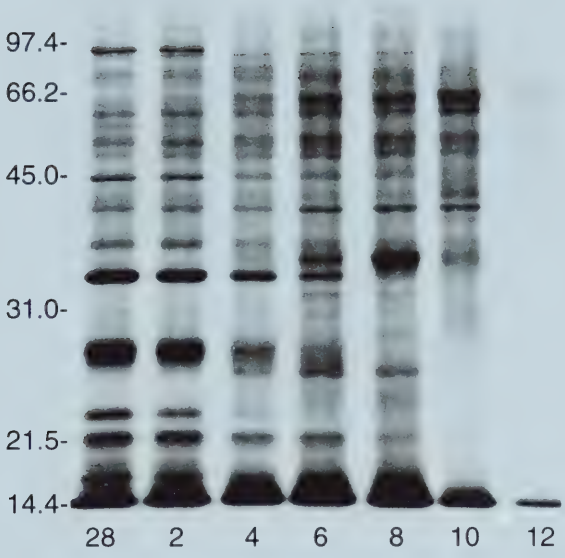
3.6.2 Qualitative changes in SDS dissociated soluble protein profile of the megagametophyte of *in vitro* cultured decoated seeds

In contrast to the seedling soluble proteins, there were noticeable changes between the megagametophyte non-reduced (Fig. 19 A) and reduced (Fig. 19 B) soluble protein profiles. In both megagametophyte reduced and non-reduced protein gel profiles the protein disappeared after 10 DIC_{27}. When not reduced, the hydrolysis of the 22 and 27.5 kDa was complete after 4 DIC_{27}. The disappearance of the 34.5 kDa protein was quite noticeable after 4 DIC_{27} and was completely hydrolyzed by 6 DIC_{27}. Complete hydrolysis of the 20 kDa and 47 kDa proteins occurred after 8 DIC_{27}. Interestingly there was the accumulation of two sets of proteins, a 25 kDa protein that started to accumulate after 4 DIC_{27} and a 34.5 kDa protein that started to accumulate after 6 DIC_{27}. However both the 20 kDa and 47 kDa polypeptides disappeared after 10 DIC_2.

When reduced, changes in the profiles of the 47 kDa, 34.5 kDa, 27 kDa, 22 kDa and 20 kDa proteins were similar to that when not reduced. However, there was the accumulation of a 61 kDa, 55 kDa, 41.5 kDa and 21 kDa polypeptides. The most noticeable change in the reduced protein profile is the rapid hydrolysis of the 34 kDa

Figure 19. Coomassie blue stained SDS-PAGE profile of phosphate buffer-soluble proteins from the megagametophyte of *in vitro* cultured decoated seed grown with intact seedling under (A) non-reducing and (B) reducing conditions. For each lane 10 µg was loaded in 10 µl for insoluble proteins. Relative molecular masses of standards in kDa, shown by numerical values adjacent to gel profiles; 28 DAI_2 seed, and seed imbibed up to 12 DAI $_{27}$.

A


97.4-
66.2-
45.0-
31.0-
21.5-
14.4-
28
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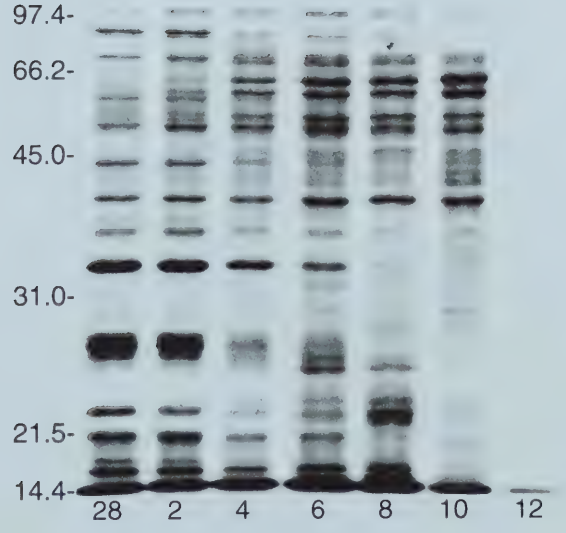
12

B


97.4-
66.2-
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31.0-
21.5-
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band by 6 DIC_{27}. The 23 kDa polypeptide was completely depleted after 2 DIC_{27} and more noticeably by 3 DAI_{27}. The 27 kDa band was also depleted by 5 DIC_{27} and another band 26 kDa started to accumulate after 4 DIC_{27}.

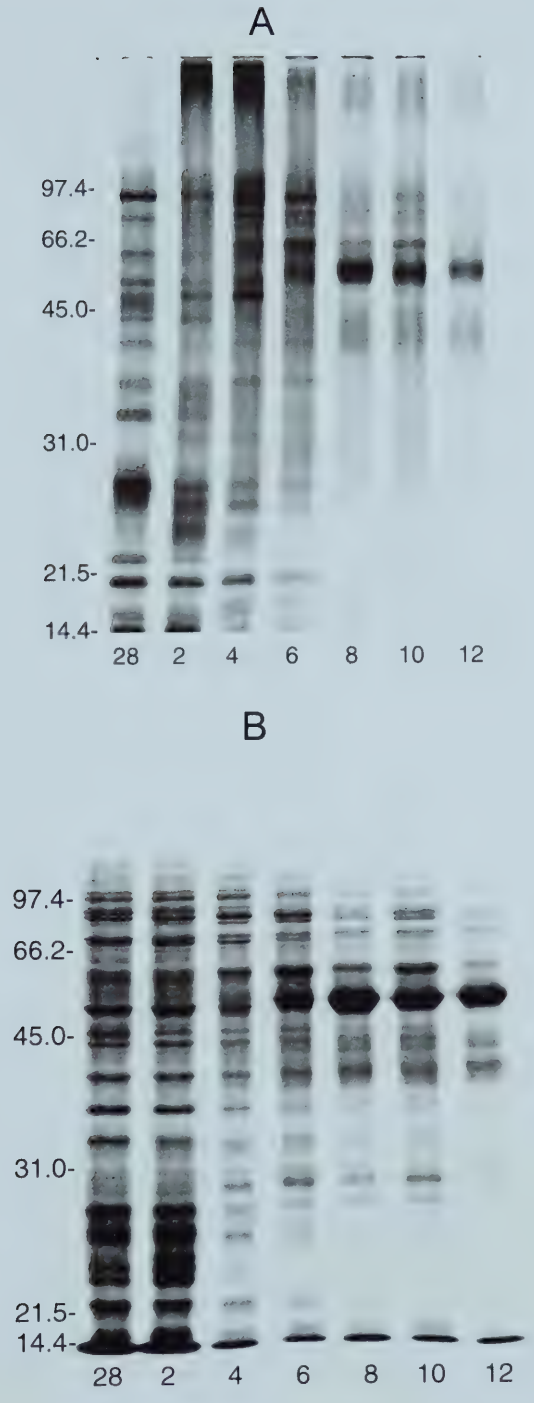
3.6.3 Qualitative changes in SDS dissociated soluble protein profile of the seedling of *in vitro* cultured decoated seeds

The pattern of protein hydrolysis of the buffer soluble SDS dissociated proteins under non-reducing and reducing conditions was very similar in both tissues during germination and early seedling growth when cultured. Fig. 20 A and 20 B shows buffer soluble protein profile obtained from the seedlings of the all the developmental stages examined. The most noticeable change was the increase in relative intensity of a 53-55 kDa protein during germination and early seedling growth. Rapid decrease in the relative intensity of the 36 kDa, 75 kDa and 27 kDa polypeptides occurred at 2 DIC_{27}. A decrease in relative intensity of the 20 and 22 kDa polypeptides was also observed at the completion of germination, however their disappearance was more gradual thereafter.

3.6.4 Qualitative changes in SDS dissociated protein profile of *in vitro* cultured isolated seedlings

Isolated seedlings where cultured at 27°C to determine the pattern of protein hydrolysis when reduced and non-reduced with the megagametophyte removed. The changes in relative intensity of the major insoluble proteins were similar to that of

Figure 20. Coomassie blue stained SDS-PAGE profile of phosphate buffer-soluble proteins from the embryo/seedling of *in vitro* cultured decoated seed grown with intact megagametophyte under (A) non-reducing and (B) reducing conditions. For each lane 10 µg was loaded in 10 µl for insoluble proteins. Relative molecular masses of standards in kDa, shown by numerical values adjacent to gel profiles; 28 DAI_2 seed, and seed imbibed up to
12 DAI $_{27}$.


A
97.4-
66.2-
45.0-
31.0-
21.5-
14.4-
28 2 4 6 8 10 12
B
97.4-
66.2-
45.0-
31.0-
21.5-
14.4-
28 2 4 6 8 10 12

seedlings cultured with the intact megagametophyte under reducing and non-reducing conditions. When not reduced, the major protein bands were the 57-58, 48-52 and 37-38 kDa and 20-22 kDa bands. By 4 DIC_{27} most of the protein bands had disappeared with the exception of the 57-58 kDa band that started to accumulate after 8 DAI_{27} over the studied period (Fig. 21 A). The relative disappearance of the 37-38 kDa and 20-22 kDa insoluble polypeptides in the reduced seedling profile (Fig. 21 B) were very similar to that of the non-reduced 57-58 kDa polypeptide. Most of the protein disappeared after 4 DIC_{27}. Hydrolysis of the 47 kDa polypeptide was similar in both reduced and non-reduced conditions and occurred by 2 DIC_{27}.

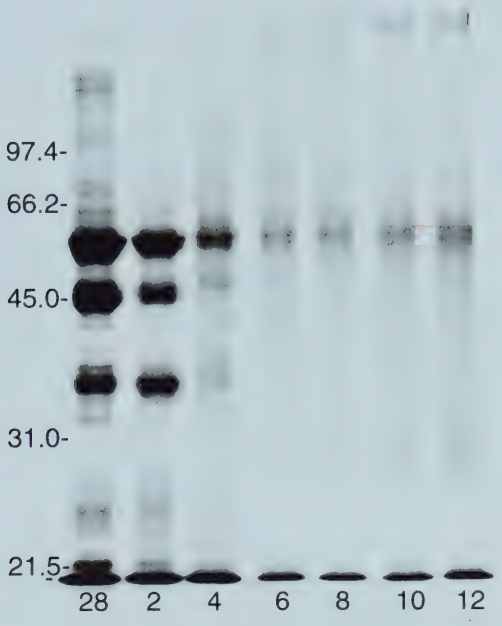
The major polypeptide bands in the reduced and non-reduced profile of isolated seedlings soluble proteins were similar in the seedlings from intact seeds. The most noticeable bands were the 73, 47, 34.5, 27.5, 22, and 20 kDa bands in both reduced and non-reduced protein profiles. However, in both non-reduced (Fig. 22 A) and reduced (Fig. 22 B) all of the protein in both the non-reduced and reduced polypeptide gel profiles disappeared after 4 DIC_{27} and started to reappear by 12 DIC_{27}.

3.7 Quantitative changes in soluble amino acid content in megagametophyte and seedling of *in vitro* cultured decoated seeds

The soluble amino acid pool in both seedlings and megagametophyte was relatively constant during germination. The soluble amino acid levels in the seedling cultured with intact megagametophytes increased rapidly after 4 DIC_{27} and peaked at

Figure 21. Coomassie blue stained SDS-PAGE profile of phosphate buffer-insoluble proteins from isolated embryo/seedling cultured *in vitro* under (A) non-reducing and (B) reducing conditions. For each lane 2.5 µg was loaded in 10 µl for insoluble proteins. Relative molecular masses of standards in kDa, shown by numerical values adjacent to gel profiles; 28 DAI_2 seed, and seed imbibed up to 12 DAI $_{27}$.

A


97.4-
66.2-
45.0-
31.0-
21.5-
28
2
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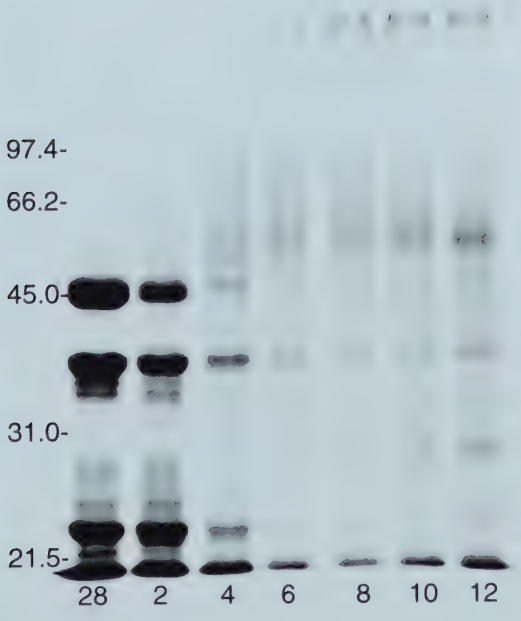
12

B


97.4-
66.2-
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10 DIC_{27} with a 260-fold increase (Fig. 23 A) and started diminishing as it reached senescence.

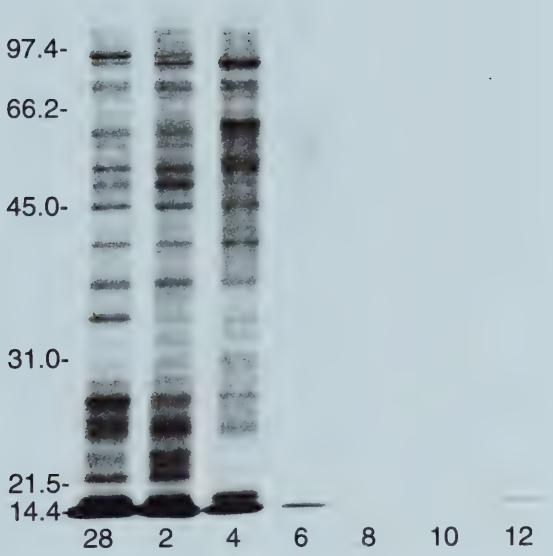
In contrast, at the completion of germination there was a 50-fold increase in the soluble amino acid pool of the megagametophytes cultured with associated seedlings (Fig. 23 B). The amino acid pool peaked at 8 DIC_{27}, and then diminished at senescence.

3.7.1 Quantitative changes in soluble amino acids content of *in vitro* cultured isolated seedlings

The soluble amino acid pool in the isolated seedlings cultured without the megagametophyte remained relatively constant until 4 DIC_{27}. There was an increase in the levels of soluble amino acid in the seedlings after 4 DIC_{27} peaking at 10 DIC_{27} with a 65-fold increase (Fig. 23 A).

Figure 22. Coomassie blue stained SDS-PAGE profile of phosphate buffer-soluble proteins from isolated embryo/seedling cultured *in vitro* under (A) non-reducing and (B) reducing conditions. For each lane 10 µg was loaded in 10 µl for soluble proteins. Relative molecular masses of standards in kDa, shown by numerical values adjacent to gel profiles; 28 DAI_2 seed, and seed imbibed up to 12 DAI $_{27}$.

A


97.4-
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21.5-
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B

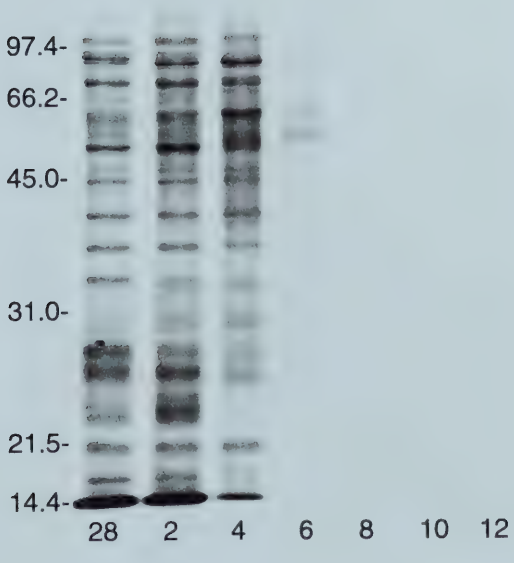
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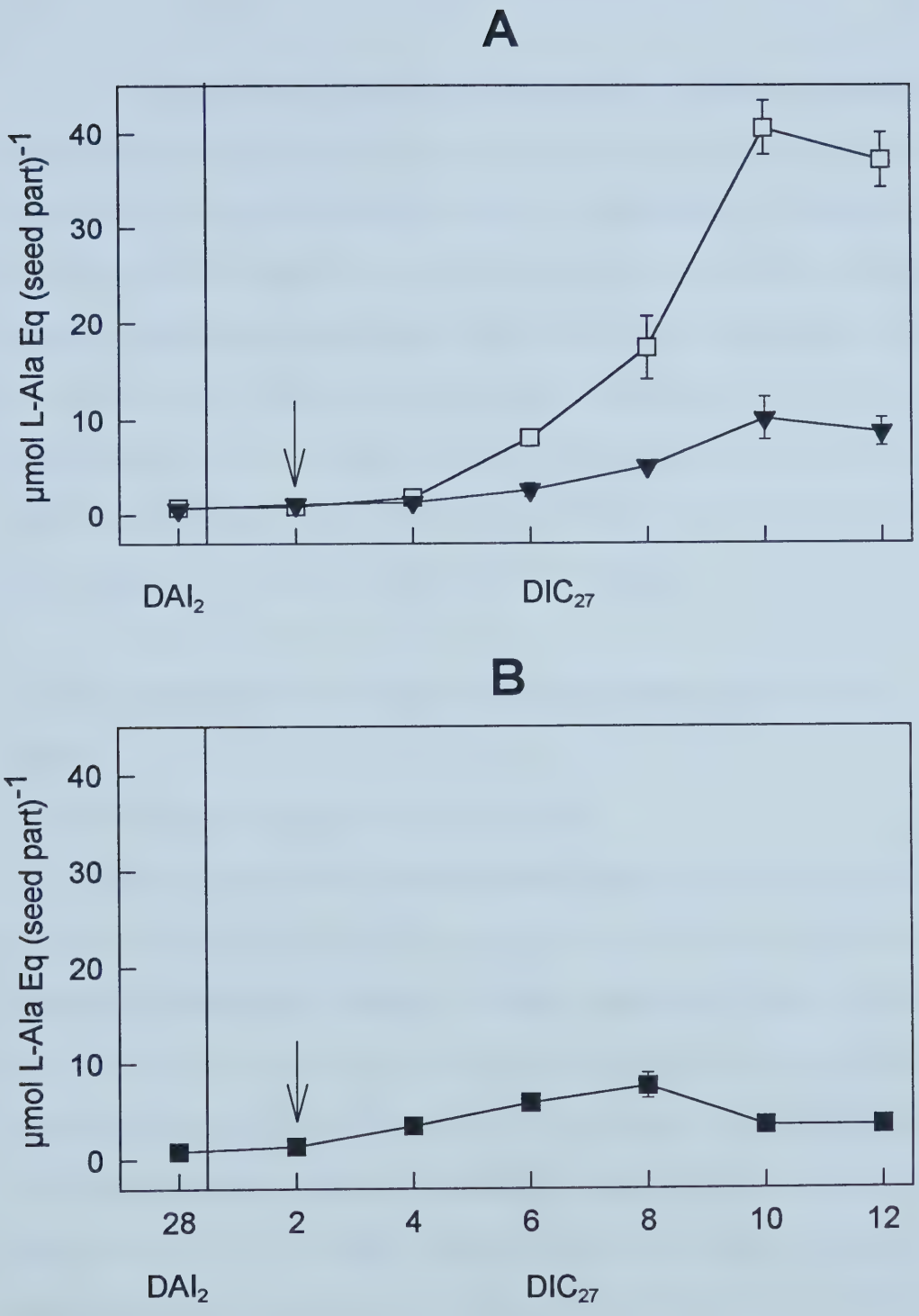
Figure 23. Quantitative changes in the soluble amino acid content from isolated seedlings and decoated seeds cultured *in vitro* at 27 °C, during germination and early seedling growth. A) Seedling; (▼) isolated, (□) intact B) Megagametophyte (■). X-axis shows fully stratified seed (28 DAI_2), and seed imbibed for up to 12 days at 27 °C. Arrow indicates the completion of germination by radicle emergence from the seed coat. Each data point is the mean of three independent biological replicates each assayed in triplicate ± standard deviation of the mean.


A
µmol L-Ala Eq (seed part)-1
40
30
20
10
0
DAI2
DIC27
B
µmol L-Ala Eq (seed part)-1
40
30
20
10
0
28
2
4
6
8
10
12
DAI2
DIC27

4.0 Discussion

The discussion section deals with the physiological and biochemical changes that occur in radiata pine seed during germination and early seedling growth. It emphasizes the relationship between the embryo and megagametophyte, characterizes the storage proteins in these tissues and explains some of the mechanisms by which these storage proteins are hydrolyzed and mobilized during early seedling growth. It also compares some of these changes in storage protein reserves to that of other plants, mainly conifers. It incorporates some of the proposed mechanisms from similar changes observed in other conifer species and their relevance to this research. Finally, questions and suggestions for future research are given.

4.1 Physiological changes in radiata pine seed during germination and early seedling growth

4.1.1 Effect of stratification on germination and growth

About 55 % of the mature radiata pine seeds used in this study were dormant and required moist treatment at 2 °C to break dormancy. This treatment period is referred to as stratification. Dormancy is an adaptive mechanism of plants in nature to ensure the survival of the species. In the laboratory and nursery, seed pretreatment is necessary to break dormancy. Some of the most commonly utilized practices to break dormancy in seeds involved the use of light, temperature, plant hormones, and osmotic pressure. Many conifer seeds are dormant and require a period of stratification at low temperature to break dormancy while others complete

germination satisfactorily without stratification. The result is an increased rate of germination and uniform growth (Stone, 1957, US Agric. Dept, 1948). In the present study, the process of stratification not only broke dormancy but also ensured that all of the seeds were fully imbibed prior to placing them at a temperature that permitted germination and seedling growth.

In radiata pine seeds, a period of 28 DAI_2 was required to break dormancy and ensure uniform and high percentage germination and seedling growth. The stratification period required to break dormancy in loblolly pine seeds is 35 DAI_2 (Schneider and Gifford, 1994) while *P monticola* is 90 days (Hoff, 1987). In the past conifer seeds were stratified at 5 °C. However, imbibition at this temperature induced the completion of germination and growth as observed in the seeds of *P. lambertiana* Dougl. and *P. jeffreyi* Amarr. Thus the practice was discontinued (Stone, 1957). Kao (1972) reported that dormancy was broken in the seeds of *P. strobus* L after 60 days of imbibition at 5.8 °C. In radiata pine, the seeds were imbibed in moisture at 2 °C for 28 days to ensure that the contribution of non-dormant seed germination during stratification was minimal.

Dormancy in conifer seeds has been attributed to a number of factors as suggested by Varnier, 1965; including the seed coat. The seed coat may render some mechanical resistance and may be impermeable to water and oxygen. Conifer seed dormancy has been attributed to the retarding effect of the seed coat to oxygen uptake (Stone, 1957). In loblolly pine seed, the megagametophyte plays a major role in the maintenance of embryo dormancy in mature seeds (King 1998). Similar observations were made for *P. monticola* (Hoff, 1987).

Another factor that may causes dormancy in conifer seeds is the presence of germination inhibitors. Hoff (1987) suggested that a number of germination inhibitors are involved. Proteinase inhibitors in resting seeds of *P. sylvestris* disappeared during stratification allowing protein hydrolysis to occur (Salmia, 1981) even though the disappearance may be due to the enzymatic breakdown and leaching out (Halmer and Bewley, 1979).

Synthesis and transportation of germination promoters may occur during the stratification period (Niko Laeva, 1968). In loblolly pine, growth regulators such as gibberellic acid and kinetin promote germination in stratified seeds (Biswas *et al.*, 1972). Paul *et al.*, (1973) found that mature loblolly pine seed contained no such growth substances, but had a high concentration of an unknown inhibitor. Following stratification, the inhibitor was undetected while high levels of gibberellic acid and auxin like substances were present. It was proposed that the synthesis of specific proteins might be needed to promote germination (Satoh and Esashi, 1979).

Germination and seedling growth rates in conifer species vary notably. However, consistencies have been observed in relation to the time the seeds complete germination that is evident by radicle emergence from the seed coat. Germination in radiata pine seeds was complete at 4 DAI_{27} *in vivo* and 2 DAI_{27} for cultured *in vitro* seeds with the seed coat removed. Sacher, 1965 reported that the growth rate of the embryos dissected from stratified and mature non-stratified radiata pine seeds cultured in Guern's medium (Guern, 1965) were the same. However, removing the seed coat reduced the time for 50 % germination from 8.3 to 7 days an insignificant change when compared to 18 to 4 days for *P. jefreyi* (Stone, 1957).

4.2 Seed storage protein reserves

In this study, the 48-52 kDa, 38-39 kDa, 20-22 kDa polypeptides form the major insoluble reserves in radiata pine megagametophyte and embryo. They are rapidly hydrolyzed after radicle emergence at 4 DAI_{27} and are similar in type and abundance to other gymnosperms species (Groome *et al.*, 1991).

These insoluble storage proteins in radiata pine seeds are similar to the glutelin-like proteins found in most conifer seeds in solubility characteristic, subunit molecular mass and subunit composition. They are characterized by their solubility in phosphate buffer containing SDS or urea (Becker *et al.*, 1978, Gifford *et al.*, 1982, Gifford and Bewley, 1983, Gifford, 1988) and are found within the amorphous matrix of the single membrane bound organelles called protein bodies of the parenchyma cells of the embryo and megagametophyte (Lott, 1980). They are actively involved in providing nutrient for the developing seedlings (Gifford *et al.*, 1982; Kermode *et al.*, 1985; Gifford 1988).

In this study, the insoluble storage proteins formed 89.4 % of the total protein in the radiata pine seed. Of the 93 % of the total storage proteins found in the megagametophyte, 93 % were insoluble in phosphate buffer. Interestingly, the megagametophyte houses 96.3 % of the insoluble proteins in the seed. The percentage of insoluble protein reserves found in the megagametophyte of radiata pine seed is relatively higher than that found in other conifer species where the proportion of insoluble proteins in the megagametophyte is between 58 to 80 % (Gifford, 1988). In lodgepole pine about 70 % of the total protein in the seed was insoluble of which 90-95 % was found in the megagametophyte (Gifford *et al.*, 1988).

The megagametophyte of loblolly pine contained 75 % of the insoluble protein (Groome *et al.*, 1991).

In radiata pine seeds, the megagametophyte and embryo insoluble protein gel profiles are similar to those observed for other pine species (Gifford, 1988). That is, the major protein comprises a 57-58 kDa polypeptide subunit that is made up of two polypeptides with respective masses of 37-41 kDa and 21-23 kDa. In addition, the 48-52 kDa polypeptide was not affected under reduced conditions and did not appear to have subunit polypeptide linked by disulphide bridges consistent with that observed for other conifer species (Gifford, 1988). The relatively high molecular mass proteins (>100 kDa) present in non-reduced gel profile of the megagametophyte and embryo of radiata pine seed are also found in other conifer species (Gifford, 1988). These protein bands disappeared under reduced conditions, which suggests that they consist of smaller subunit polypeptide linked by disulphide bridges. Since glutellin-like storage proteins have quaternary structures these high molecular mass proteins may possibly come from the same origin (Bewley and Black, 1994).

The soluble proteins from the megagametophyte and embryo of different conifer species show much variation. One exception is a low molecular mass protein in the range of 27-29.5 kDa that has storage function and present in many of the conifer species studied to date (Gifford, 1988, Gifford and Lammer, 1989). This corresponds to the 27.5 kDa protein band present in the soluble protein profile of radiata pine seed. Two other major proteins of radiata pine that appear to have a storage function are the 22 kDa and 34.5 kDa polypeptides. Like most other conifer

species reduction did not affect the major soluble proteins of radiata pine seed megagametophyte and embryo.

Although the insoluble proteins form the bulk of the storage reserves in most conifers, in *P.albicaus, P. glauca* and some species of *Abies* the soluble proteins are the major storage reserves (Gifford, 1988; Gifford and Tolley, 1989; Kovac and Kregar, 1989; Jensen and Lixue, 1991). In white spruce the insoluble proteins accounted for 20-25 % of the total protein (Gifford and Tolley, 1989). Insoluble storage proteins have also been identified in angiosperm seeds such as Euphorbiaceae and Cucurbitaceae (O'Kennedy *et al.*, 1979; Lalonde *et al.*, 1984).

4.3 Storage protein hydrolysis

Germination and early seedling growth is characterized by both biochemical and physiological changes (Bewley and Black, 1994). These changes usually involve hydrolysis of storage protein reserves to soluble amino acids before they can be mobilized to the developing seedling were they are utilized as energy source for the developing seedling (Bewley and Black, 1978).

In radiata pine, the free amino acids generated by the hydrolysis of the megagametophyte storage proteins appeared to be exported immediately to the developing embryonic axis following their production. Cultured seedlings without associated embryo mobilized their storage protein reserves rapidly after the completion of germination, however this was not accompanied by an increase in the free amino acid pool. It is evident that the increase in free amino acid pool in the seedlings cultured with associated megagametophyte was not a direct result of

embryonic storage protein hydrolysis alone but was also due to importation of the amino acids from the megagametophyte. This is similar to lodgepole pine and loblolly pine (Lammer and Gifford, 1989; Groome *et al.*, 1991). The embryo mobilizes its own protein reserves initially as shown by the rapid insoluble protein depletion before radicle emergence and then depends on the megagametophyte for amino acids. Cardinil and Reinero (1982) noted that the megagametophyte is not the main source for nutrients prior to radicle emergence. In many other conifers the embryo reserve begins hydrolysis before any similar pattern in the megagametophyte are detected (Simola 1974; Kovac and Kregar, 1989). The embryo can develop into a seedling by relying exclusively on its own reserves as shown in the isolated seedlings cultured *in vitro* even though the megagametophyte plays a role later in its development.

4.4 Future Research

Although progress has been made towards understanding the mobilization of storage protein in germinated seeds a clear understanding of the processes involved has not yet been achieved. In this study, I demonstrated that storage proteins reserves of radiata pine seeds are hydrolyzed during germination and early seedling growth. The constituent amino acids are then mobilized from the megagametophyte to the seedling where they are utilized as carbon and nitrogen sources for the growing seedling until it becomes autotrophic. Future studies will focus on the enzymes involved in the breakdown of seed storage reserves and on mechanisms involved in the transport of amino acids.

In relation to this, the cloning of the genes that code for the enzymes and developmental regulation of these genes also need to be studied. Also, we need to determine the role of the embryo in regulating reserve mobilization in the megagametophyte. This could be facilitated by the availability of antibodies and cDNA probes. Because of the similarities in the pattern of development among conifer species, results on the control of reserve mobilization in radiata pine system will significantly increase the understanding of conifer germination and early seedling growth.

The results of this research provide basic information on the storage protein reserves breakdown. This study serves as an information base for future studies on the molecular and biochemical events during radiata pine seed germination. Results from this study will be valuable in improving *in vitro* propagation of radiata pine for commercial purposes where knowledge of the storage reserves and their mobilization is important.

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